Role of Kappa-Opioid Receptors in Stress-Induced Behaviors

Dissertation Advisor: Dr. William A. Carlezon Jr. Ashlee Victoria Van't Veer

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Abstract

The development of anxiety and mood disorders often coincides with exposure to stress. Accumulating evidence indicates that both corticotropin-releasing factor (CRF) and dynorphin, the endogenous ligand for the kappa-opioid receptor (KOR), can mediate the effects of stress. My dissertation research utilized laboratory animals to investigate the role of KORs in stress-induced increases in the acoustic startle response, a metric often used to study stress effects in humans. Using wild-type mice, I first demonstrated that systemic administration of a KOR antagonist produced an anxiolytic-like effect on acoustic startle following central (intracerebroventricular) infusion of CRF. Immunohistochemical analysis revealed that KOR blockade decreased c-Fos cell counts in the dentate gyrus of the hippocampus in both vehicle- and CRF-treated mice, and reduced CRF-induced increases in the ventral tegmental area (VTA). Within the VTA, reductions were predominantly in dopaminergic neurons. KOR antagonist pretreatment also produced anxiolytic-like effects on footshock-potentiated startle, a model that quantifies context-specific fear conditioning. To complement the antagonist studies, we developed constitutive knockout mice that lack KORs throughout the brain ($KOR^{-/-}$), and conditional KOs that lack KORs only within dopaminergic neurons ($DAT\text{-}KOR^{lox/lox}$). Initial characterization demonstrated that these two mutant lines did not differ from controls in hearing, vision, weight gain, and

locomotor activity. $KOR^{-/-}$ mice were similar to controls in unconditioned anxiety-like behavior, but DAT-$KOR^{lox/lox}$ mice displayed nominal decreases in anxiety-like behavior in the open field and light/dark box. Unexpectedly, KOR ablation did not affect CRF-induced increases in startle in either mutant line. Importantly, however, KOR antagonist treatment did not alter CRF-induced increases in startle in $KOR^{-/-}$ mice, suggesting that KOR antagonist effects in wild-type mice are due to blockade of KORs. These findings raise the possibility that differences in KOR antagonist and $KOR^{-/-}$ studies may be related to brief KOR blockade during adulthood versus a lack of KORs during the entire lifespan. In the footshock-potentiated startle paradigm, $KOR^{-/-}$ mice were comparable to littermate controls, whereas DAT-$KOR^{lox/lox}$ mice showed attenuated effects of footshock. My findings confirm a role for KORs in fear and anxiety-like behavior in rodents, and implicate KORs expressed on dopaminergic neurons in modulating important aspects of stress-related behavior.

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Abbreviations

5CSRTT	five-choice serial reaction time task
AAV	adeno-associated virus
ABC	avidin-biotin complex
aCSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
AMY	amygdala
ASR	acoustic startle reflex
AVP	arginine vasopressin
BLA	basolateral nucleus of the amygdala
BNST	bed nucleus of the stria terminalis
bp	base pair
CeA	central nucleus of the amygdala
CNS	central nervous system
CPP/A	conditioned place preference/aversion
Cre	cre-recombinase
CREB	cAMP response element binding protein
CRF	corticotropin-releasing factor
CRF-BP	corticotropin-releasing factor binding protein
CRF-R	corticotropin-releasing factor receptor
CRN	cochlear root neurons
CS	conditioned stimulus
CSDS	chronic social defeat stress
DA	dopamine
DAB	3,3'-diaminobenzidine
DAT	dopamine transporter
dB	decibel
DBS	deep brain stimulation
DRN	dorsal raphe nucleus
EPM	elevated plus maze
ERK	extracellular regulated kinase
ES	embryonic stem
FST	forced swimming test
GABA	gamma-aminobutyric acid
GIRK	G protein-coupled inwardly-rectifying potassium channel
GPCR	G protein-coupled receptor
GR	glucocorticoid receptor
HIP	hippocampus
HPA	hypothalamic-pituitary-adrenal axis
ICSS	intracranial self-stimulation
ICV	intracerebroventricular
IEG	immediate early gene
IP	intraperitoneal
ISI	interstimulus interval
ITC	intercalated cell

JNK	c-Jun N-terminal kinase
KO	knockout
KOR	kappa-opioid receptor
MAPK	mitogen-activated protein kinase
MAOI	monoamine oxidase inhibitor
MOR	mu-opioid receptor
MR	mineralocorticoid receptor
NAc	nucleus accumbens
ND	not detectable
NGS	normal goat serum
No stim	no stimulus
NorBNI	nor-binaltorphimine
NS	not significant
OCD	obsessive compulsive disorder
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pdyn	prodynorphin
PFC	prefrontal cortex
PnC	caudal nucleus of the pontine reticular formation
PTSD	post-traumatic stress disorder
PVN	paraventricular nucleus of the hypothalamus
qPCR	quantitative reverse transcriptase polymerase chain reaction
SEM	standard error of the mean
SI	social interaction
SN	substantia nigra
SNRI	serotonin and norepinephrine reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
TCA	tricyclic antidepressant
TH	tyrosine hydroxylase
UCN	urocortin
US	unconditioned stimulus
Veh	Vehicle
VTA	ventral tegmental area
WT	wild-type

Chapter 1

Introduction

Anxiety disorders represent an increasingly prevalent form of psychiatric illnesses, the effects of which can be debilitating. The neurobiological basis of these disorders is not understood. When functioning normally, the stress response is an adaptive process that maintains homeostasis. However, prolonged stress or over-activation of the stress response is implicated in the development of myriad health issues including anxiety and depressive disorders (Kessler, 1997; Heim and Nemeroff, 1999). Corticotropin-releasing factor (CRF) is an important mediator of the stress response, and this peptide regulates hormonal, autonomic, and behavioral consequences of stress (Dunn and Berridge, 1990; Bale et al., 2002). In animals, CRF induces a stress-like state when administered into the central nervous system (CNS) (Sutton et al., 1982) and leads to behavioral signs analogous to those seen in people with depressive and anxiety disorders (Dunn and Berridge, 1990; Owens and Nemeroff, 1991; Koob et al., 1993), suggesting that it plays an important role in the negative consequences of stress. Indeed, dysregulation of CRF function is thought to underlie human depressive and anxiety disorders (Nemeroff, 1992; Owens et al., 1993; De Souza, 1995). As such, investigations into the activation of CRF signaling and downstream effects may provide insight into the mechanisms by which stress can trigger psychiatric illness.

Accumulating evidence links the kappa-opioid receptor (KOR) system with CRF-induced effects. This system comprises KORs and their endogenous ligand dynorphin (Chavkin et al., 1982). Like activation of the CRF system, activation of the KOR system has been implicated in the negative consequences of stress in rodents such as learned helplessness, drug seeking, and memory deficits (Pliakas et al., 2001; Newton et al., 2002; Mague et al., 2003; McLaughlin et al., 2003a; McLaughlin et al., 2006b; Carey et al., 2009). Recently, it has been demonstrated in mice that CRF-induced aversion and anxiety-like behavior are dependent on dynorphin and KOR activation (Bruchas et al., 2009; Land et al., 2009; Knoll et al., 2011). These effects were attributed to KOR signaling in the dorsal raphe nucleus (DRN) and amygdala (AMY) respectively. These regions work within a network of interconnected structures, which together contribute to behavioral output.

The mesocorticolimbic system, comprising the ventral tegmental area (VTA) and its dopaminergic outputs to the nucleus accumbens (NAc), hippocampus (HIP), bed nucleus of the stria terminalis (BNST), prefrontal cortex (PFC), and AMY is one neural circuitry that integrates CRF and KOR effects. Each component of this system has been implicated in stress and co-expresses CRF receptors (CRF-Rs) and KORs (De Souza et al., 1985; Mansour et al., 1995; Dautzenberg and Hauger, 2002). Although the mesocorticolimbic system has not traditionally been studied for its role in depression and anxiety (Nestler and Carlezon, 2006), it is becoming increasingly clear that this circuit is involved in key aspects of these disorders (Pliakas et al., 2001; Barrot et al., 2002; Barrot et al., 2005). Antagonism of KORs within the NAc reduces depressive-like behavior (Newton et al., 2002; Shirayama et al., 2004) while NAc infusions of a KOR

agonist into this region induce anhedonia (Muschamp et al., 2011b), a core symptom of depression. Anxiety disorders are highly comorbid with depression, as over half of patients with lifetime major depression also meet the criteria for an anxiety disorder (Kaufman and Charney, 2000; Kessler et al., 2003). Many treatments for depression are also effective in treating anxiety disorders (Ballenger, 1999), suggesting common genetic or neuroanatomic components. As such, the mesocorticolimbic system may be a site of KOR activation that plays an important role in regulation of both depressive- and anxiety-like behavior. *The goals of this dissertation work are to further delineate the general role of KORs in mouse models of stress-induced behaviors, and to test the hypothesis that KORs within the mesocorticolimbic system play a particularly important role in regulating anxiety-related behavior*. An increased understanding of the systems mediating stress-induced illness may provide new opportunities for developing improved treatments for anxiety disorders.

This chapter first provides background on stress and introduces CRF and its role in anxiety-and depressive-like behaviors. The KOR system is introduced with focus on its role in stress-induced behavior and interactions with CRF. The neural circuits underlying stress and anxiety are then described, with particular emphasis on the VTA and its target structures. The chapter ends with a summary of the primary behavioral paradigms used to investigate the hypothesis that stress-related activation of KORs within the mesocorticolimbic system mediates anxiety-like behaviors as measured by an increase in the acoustic startle response.

1. Anxiety

Mental illnesses are the most common health problem today, with up to half of Americans meeting the diagnostic criteria for a disorder at some point in their lifetime. Anxiety disorders have a lifetime prevalence of 28.8% (Kessler et al., 2005b) and affect more than 40 million Americans in a given year (Kessler et al., 2005a). Signs (objective metrics visible to an observer) and symptoms (subjective metrics described by the patient) of anxiety disorders include feelings of apprehension or dread, trouble concentrating, excessive worrying, insomnia, and pounding heart. These effects make anxiety disorders the leading cause of workplace disability in the United States (Kessler, 2000) and cost America an estimated $42.3 billion in 1990 (Greenberg et al., 1999), a price that has been increasing each year. The fact that the neurobiology of anxiety disorders is poorly understood contributes to difficulties in the development of new treatments that are safer and more effective than present medications, which tend to have many side effects including sedation and abuse liability (Woods et al., 1992). Because stress is a major trigger for psychiatric disorders including anxiety, new insights might be gained from the study of stress-regulated neural circuits.

2. Background on stress systems

2.1 Effects of stress

Endocrinologist Hans Selye defined stress as “the rate of wear and tear in the body” (Selye, 1956). The concept of stress focuses on the notion that internal or external factors can negatively impact psychological and physical well-being. Acutely, stress leads to involuntary hormonal (e.g., increased free fatty acid generation, inhibition of the immune system), autonomic (e.g., increased heart and breathing rate, increased blood

flow to the brain and muscle), and behavioral (e.g., feelings of anxiety and fear, heightened vigilance) changes—often collectively called “the stress response”—that prepare the body to maintain homeostasis in response to a real or perceived threat. This adaptive response is generally protective in the short term (Keay and Bandler, 2001). However, severe or sustained stress often coincides with development of anxiety disorders, clinical depression, and drug abuse (Kessler, 1997; Kendler et al., 1999; Pine et al., 2002; Volkow and Li, 2004; Fox et al., 2007; Koob and Kreek, 2007). In laboratory settings, stress paradigms often trigger depressive- and anxiety-like behaviors in model organisms. Discrete types of stress (stressors) including footshock, maternal deprivation, and restraint induce depressive-like behaviors (e.g., increased immobility in the forced swimming test [FST]) (Platt and Stone, 1982; Aisa et al., 2008) and elevations in brain reward thresholds (Zacharko and Anisman, 1991). More natural stressors also produce similar outcomes: as an example, subordinate mice in a chronic social defeat stress (CSDS) paradigm—an ethologically relevant stressor involving daily exposure to an aggressor—show anxiogenic-like responses such as spending less time in the lit area of a light/dark box and the open arms of an elevated plus maze (EPM) (Keeney and Hogg, 1999; Slattery et al., 2012), as well as decreases in social interaction (SI) with other mice (Avgustinovich et al., 2005; Berton et al., 2006). Other ethologically-relevant forms of stress, such as resident-intruder stress and neonatal stress induced by maternal separation, also subsequently produce depressive-like effects in the FST (Wood et al., 2012), as well as in the SI test (Aisa et al., 2008; Marco et al., 2009). These studies demonstrate how models of stress in rodents may enable valuable insights into the mechanisms of stress-induced illness in humans.

2.2 Corticotropin-releasing factor (CRF)

CRF, first described by Vale and colleagues (1981), is the principal regulator of the stress response (Spiess et al., 1981; Majzoub, 2006). It is part of a family of peptides in mammals also including urocortin 1 (UCN1), urocortin 2 (UCN2) and urocortin 3 (UCN3) (for review, see Reul and Holsboer, 2002). The actions of these peptides are mediated through two cognate G-protein-coupled receptors, CRF-R1 and CRF-R2 (for review, see Dautzenberg and Hauger, 2002; Eckart et al., 2002). CRF can activate both receptors, although it has greater affinity for CRF-R1 over CRF-R2. UCN1 can also activate CRF-R1, whereas CRF-R2 binds all UCNs with high affinity. CRF-Rs are predominantly linked to the G_s-alpha subunit, which activates adenylate cyclase and cAMP-dependent pathway signaling. However, CRF-Rs also appear capable of coupling to multiple other G proteins, including G_i and $G_{q/11}$, and thus can regulate multiple signaling cascades (Grammatopoulos et al., 2001; Blank et al., 2003) in a brain region-specific manner.

CRF produced by cells in the paraventricular nucleus of the hypothalamus (PVN) triggers hormonal stress responses by activating the hypothalamic-pituitary-adrenal (HPA) axis, which leads to the release of adrenocorticotropic hormone (ACTH). In turn, ACTH stimulates glucocorticoid release from the adrenal glands, which produces subsequent metabolic and cardiovascular changes. Both CRF-containing neurons and CRF receptors are found throughout brain regions implicated in behavioral and autonomic responses, suggesting that CRF can also have direct effects in these regions, independent of HPA axis activation (Swanson et al., 1983; Merchenthaler, 1984; Sakanaka et al., 1987; Koob et al., 1993; Dautzenberg and Hauger, 2002). Central CRF administration in laboratory animals has been shown to produce

autonomic and behavioral effects that mimic acute stress responses, including increased heart rate and arterial pressure, and decreased food intake and reproductive behavior (Dunn and Berridge, 1990). However, the downstream effects following receptor activation are not fully understood. Further investigation into the systems that are responsive to CRF may provide new insights into how stress affects behavior.

2.2.1 CRF in depression and anxiety

CRF is also implicated in the detrimental consequences of prolonged stress. Indeed, hypersecretion of CRF has been hypothesized to be the primary contributing factor in the development of depressive and anxiety disorders (Nemeroff, 1992; Owens et al., 1993; De Souza, 1995). In humans, major depressive disorder has been associated with higher levels of cerebrospinal fluid CRF (Nemeroff et al., 1984; Widerlov et al., 1988; Arato et al., 1989; Kasckow et al., 2001), and CRF levels are also elevated in patients with post-traumatic stress disorder (PTSD) (Bremner et al., 1997a; Baker et al., 1999).

CRF-Rs can be found throughout the brain, including within elements of the mesocorticolimbic system (e.g., VTA, NAc, AMY) (De Souza et al., 1985; Millan et al., 1986; Van Pett et al., 2000; Dautzenberg and Hauger, 2002). Given this distribution, CRF can directly modulate brain circuits implicated in anxiety and depressive responses. Exogenous administration of CRF induces anxiety and depressive-like behavior in laboratory animals, enabling studies of cause-effect relationships between stress and behaviors that reflect the signs of psychiatric illness. For example, socially housed nonhuman primates exhibit depressive-like behaviors such as huddling and

wall-facing after intracerebroventricular (ICV) CRF infusion (Kalin, 1990; Strome et al., 2002). In rodents, CRF also precipitates depressive- and anxiety-like behaviors such as increased immobility in the FST, suppression of exploratory behavior in a novel environment, potentiation of acoustic startle responses, and reduction of social interaction (Britton et al., 1982; Dunn and File, 1987; Liang et al., 1992; Swiergiel et al., 2008). Similarly, mice overexpressing CRF throughout their lifespan show evidence of anxiety-like behavior such as decreased open arm time in the EPM, reduced time in the light compartment of a light/dark box, and decreased locomotor activity in their home cage (Stenzel-Poore et al., 1994; van Gaalen et al., 2002). Another way of elevating CRF levels is through knockout (KO) of CRF binding protein, which normally binds free CRF and inactivates it. Similar to the effects of administration and overexpression of CRF, ablation of CRF-BP produces an anxiogenic-like phenotype in the EPM (Karolyi et al., 1999). Together, these findings provide strong evidence for a role of CRF in the development of stress-induced illness.

In contrast to the effects of CRF-R activation, CRF-R antagonists have antidepressant effects in humans and antidepressant-like effects in laboratory animals (Zobel et al., 2000; Griebel et al., 2002). In non-human primates, administration of the CRF-R1 antagonist antalarmin decreases anxiety and fear behavior and increases exploratory and sexual behavior when animals are exposed to stressful stimuli (Habib et al., 2000). CRF-R1 antagonists also reduce anxiety-like responses in rodents (Britton et al., 1986; Deak et al., 1999), and rats infused with CRF-R1 antisense oligonucleotides display anxiolytic-like behavior in the EPM following social defeat (Liebsch et al., 1999). Similarly, mice lacking CRF-Rs display decreased anxiety-like behavior indicated by

more time spent in the open arms of the EPM and light compartment of the light/dark box compared to controls (Smith et al., 1998; Timpl et al., 1998). The role of CRF-R2 in anxiety is less clear, since there have been reports of both increases and decreases in anxiety-like behaviors mediated through this receptor. For example, selective systemic activation of CRF-R2 with UCN3 in rats resulted in increased open arm exploration (an anxiolytic-like effect) in the EPM (Valdez et al., 2003), whereas CRF-R2 KO mice show increased anxiety in both the EPM and open-field tests (Bale et al., 2000; Kishimoto et al., 2000). However, UCN2 activation of CRF-R2 in mice increased anxiety-like behavior in the EPM, and a selective CRF-R2 antagonist reduced anxiety-like behavior (Pelleymounter et al., 2002). This apparent discrepancy may be due to differences in CRF-R2 actions depending upon species, brain region, or cell type.

A connection between CRF and mood disorders has also been established by examining the effects of standard antidepressant drugs. In rats, chronic treatment with the tricyclic antidepressant (TCA) imipramine decreased levels of CRF mRNA within the PVN (Brady et al., 1991). Similar effects were produced by antidepressant drugs with different mechanisms of action, including fluoxetine (a selective serotonin reuptake inhibitor [SSRI]) and phenelzine (a monoamine oxidase inhibitor [MAOI]) (Brady et al., 1992; Fadda et al., 1995; Aubry et al., 1999). Interestingly, in studies where short-term treatments were also investigated—regimens that do not produce full therapeutic efficacy—these drugs did not affect CRF mRNA (Brady et al., 1991; Brady et al., 1992; Aubry et al., 1999), suggesting antidepressant effects may rely at least in part by reductions of CRF in the brain. In addition to producing changes in baseline CRF levels, antidepressants also attenuate the effects of stress on CRF. Stress-induced

increases in CRF RNA in the PVN were reduced by venlafaxine, a serotonin and norepinephrine reuptake inhibitor (SNRI), and tranylcypromine, an MAOI. Furthermore, chronic stress-induced increases in CRF mRNA in the PVN were blocked by chronic venlafaxine (Stout et al., 2002). In humans, it was shown that an antidepressant reduces cerebrospinal fluid CRF levels compared to pretreatment levels in treatment responders (Heuser et al., 1998). These data suggest standard antidepressants, despite having myriad mechanisms of action, may share a common ability to decrease stress-induced CRF mRNA production. Likewise, CRF mRNA is also decreased by anxiolytic drugs (Skelton et al., 2000). These studies collectively demonstrate a link between CRF and depression and anxiety, and provide a strong rationale for studies of CRF as a method to provide new insights on the affective consequences of stress. Understanding the systems downstream of CRF-R activation may promote an improved understanding of the progression from stress exposure to states of disease and illness.

3. Kappa-opioid receptor (KOR) system

3.1 KOR localization

Endogenous opioid systems play an important role in stress, reward processing, and mood regulation. These systems consist of the neuropeptides endorphins, enkephalins, and dynorphins and their cognate receptors (mu, delta and kappa respectively). Biologically active peptides for all receptors are derived from inactive prohormones that are post-translationally processed. The dynorphin family of peptides derives from processing of prodynorphin (Pdyn) into seven major products (see Bruijnzeel, 2009; Schwarzer, 2009) that preferentially bind to and activate KORs (Chavkin et al., 1982),

although with differing potency (James et al., 1984). These dynorphin peptides, along with KORs, make up the KOR system.

The brain distribution of dynorphin has been assessed by quantifying mRNA and peptide signals in mammals including rat, mouse, and human. These studies indicate high overlap across species. Moderate to high levels of dynorphin mRNA expression have been detected in brain areas implicated in depression and anxiety including the PVN, AMY, HIP, BNST and NAc of rodents (Morris et al., 1986; Merchenthaler et al., 1997; Lin et al., 2006). A similar expression profile exists in human brain (Simonin et al., 1995; Zhu et al., 1995; Hurd, 1996; Nikoshkov et al., 2005), suggesting the KOR system plays a conserved role in rodents and humans. Studies of dynorphin protein localization demonstrate that expression overlaps with Pdyn mRNA-positive regions and their targets (Khachaturian et al., 1982; Vincent et al., 1982; Weber et al., 1982; Weber and Barchas, 1983; Code and Fallon, 1986; Fallon and Leslie, 1986) confirming that mRNA is translated into dynorphin peptides throughout the brain, particularly in stress-responsive brain regions.

There is a tight correspondence between dynorphin and KOR localization. KOR mRNA is expressed at high levels in the VTA, substantia nigra (SN), NAc, caudate putamen (CPu), hypothalamus, and AMY (Meng et al., 1993). This is consistent, for the most part, with receptor autoradiography indicating high levels of binding in the NAc, CPu, hypothalamus and AMY (Mansour et al., 1987, 1988). However, an exception to this general rule is observed within the midbrain: there is high expression of KOR mRNA in the VTA and SN despite low levels of KOR expression in these areas. It is proposed

that KORs are synthesized in the VTA and SN, and then transported via receptor trafficking to terminals in the NAc and CPu, respectively (Mansour et al., 1995). Indeed, electron microscopic immunohistochemistry has shown KORs localized to presynaptic dopamine (DA) terminals in dynorphinergic regions where they are involved in the regulation of DA transmission (Donzanti et al., 1992; Svingos et al., 1999). Given this distribution, KOR activation has the potential to modulate mesocorticolimbic circuits involved in stress and anxiety, as will be described in more detail below.

3.2 KOR signaling

KORs are G protein-coupled receptors (GPCRs) that mainly interact with inhibitory G_{α} subunits (Law et al., 2000). Activation of KORs by endogenous or synthetic agonists produces inhibition of adenylate cyclase activity as seen in cell lines (Lawrence and Bidlack, 1993), spinal cord (Attali et al., 1989) and brain membranes including striatum, HIP, AMY and cerebellum (Konkoy and Childers, 1989, 1993). KOR agonists can also decrease cell excitability and neurotransmitter release through G proteins by inhibiting voltage-gated calcium currents (Gross et al., 1990; Tallent et al., 1994; Rusin et al., 1997; Hjelmstad and Fields, 2003) or activating G protein-coupled inwardly-rectifying and voltage-dependent potassium channels (Henry et al., 1995; Simmons and Chavkin, 1996). KOR activation has also been shown to inhibit phospholipase C (Misawa et al., 1990) and activate mitogen activated protein kinase (MAPK) pathways in neurons and astrocytes (Belcheva et al., 2005). The MAPK family includes several kinases that respond to a variety of cell stimuli. These pathways regulate diverse functions such as proliferation, differentiation, apoptosis, and gene expression. MAPK activation by KORs has been shown to occur through G protein-dependent and -independent mechanisms.

For example, extracellular-signal related kinase (ERK) activation (Belcheva et al., 1998; Bohn et al., 2000; Belcheva et al., 2005) and c-Jun N-terminal kinase (JNK) (Kam et al., 2004) were shown to be G protein-dependent while p38 MAPK increases were arrestin-dependent but G protein-independent (Bruchas et al., 2006). This variety of KOR effectors enables differential responses according to receptor location, microenvironment, and binding partners. These signaling mechanisms allow for rapid effects on cell excitability and transmitter release that may underlie acute stress effects, as well as delayed effects such as gene expression which may play a role in conditions of chronic stress (Knoll and Carlezon, 2010).

3.3 Behavioral effects of KOR agonists and antagonists

KOR agonists are similar to other opioid agonists in that they produce analgesia (Pasternak, 1980; McLaughlin et al., 2004), but have reduced abuse potential as compared to mu-opioid receptor (MOR) agonists (Fraser and Rosenberg, 1964; Tang and Collins, 1985). Consequently, KOR agonists seemed ideally suited as a non-addictive pain treatment. Unfortunately, studies in humans revealed that selective KOR agonists produced dysphoria, anxiety, and abnormal behavior along with psychotomimesis at higher doses (Pfeiffer et al., 1986), which hindered their development as therapeutics (Carlezon et al., 2009). These KOR agonist-induced psychological effects are in opposition to the euphoria induced by MOR agonist, suggesting an antagonistic role of the KOR system in the brain—an “anti-reward” system—to that of MORs (Koob and Le Moal, 2008). For example, when rodents are trained to associate a KOR agonist with a distinct chamber of a multi-chamber place conditioning apparatus, they later avoid this chamber when allowed access to the entire

apparatus (Iwamoto, 1985; Shippenberg and Herz, 1987; Zhang et al., 2005). This type of conditioned place avoidance (CPA) behavior is interpreted as evidence for a drug-induced negative mood (aversive) state (Carlezon, 2003). In contrast, MOR agonists produce conditioned place preference (CPP) in that rodents prefer the drug paired side and spend more time in this chamber during the test session (Rossi and Reid, 1976). KOR agonists also elevate brain reward thresholds (Todtenkopf et al., 2004; Carlezon et al., 2006; Tomasiewicz et al., 2008; Dinieri et al., 2009), indicating anhedonia, and increase immobility in the FST (Mague et al., 2003; Carlezon et al., 2006), an effect that is opposite to that of antidepressants (Porsolt et al., 1977a; Detke et al., 1995) and often interpreted to indicate a prodepressive-like effect. These KOR agonist effects resemble behaviors observed following stress or CRF treatment, suggesting a common mechanism of action.

KOR agonists also attenuate the effects of drugs of abuse including cocaine, nicotine, and amphetamine, further supporting the idea of a KOR mediated “anti-reward” system. KOR agonists block the activating and rewarding effects of cocaine (Suzuki et al., 1992; Heidbreder et al., 1993; Heidbreder et al., 1995; Zhang et al., 2005), attenuate cocaine-induced decreases in brain reward thresholds (Tomasiewicz et al., 2008; Dinieri et al., 2009), and reduce cocaine intravenous self-administration (Glick et al., 1995; Negus et al., 1997). It is thought that rewarding properties of drugs of abuse are attributable to their common ability to elevate extracellular concentrations of DA in the NAc (Wise and Rompre, 1989); therefore, reducing DA transmission may decrease their acute effects and abuse liability. Drugs of abuse also increase release of striatal dynorphin (Hanson et al., 1988; Sivam, 1989; Smiley et al., 1990) which may modulate DA release through

presynaptic activation of KORs in the NAc (Svingos et al., 1999) to counteract the rewarding effects of drugs. Indeed, systemic and intra-NAc administration of KOR agonists decrease DA levels in the NAc (Di Chiara and Imperato, 1988; Spanagel et al., 1992; Carlezon et al., 2006) which is also postulated as a mechanism for their aversive effects (Carlezon and Thomas, 2009). Interestingly, similar to drugs of abuse—but opposite to KOR agonists—both stress and anxiogenic drugs can increase DA in the NAc; however, chronic or intense stress can decrease DA in the NAc and inhibit CRF-induced increases (Abercrombie et al., 1989; Sorg and Kalivas, 1991; McCullough and Salamone, 1992; Imperato et al., 1993; Young et al., 1993; Tidey and Miczek, 1996; Ghiglieri et al., 1997; Gambarana et al., 1999; Yadid et al., 2001; Lemos et al., 2012b). This change corresponds with a switch from appetitive to aversive responses following NAc CRF infusions, and may represent one aspect of the mechanism for the induction of depressive-like behavior following stress (Lemos et al., 2012b).

Studies involving pharmacological blockade of KOR signaling have predominantly used three selective KOR antagonists: nor-binaltorphimine (norBNI), GNTI (a norBNI derivative), and/or JDTic, a structurally dissimilar compound (Béguin and Cohen, 2009). These antagonists all share the unique ability to antagonize KORs in vivo for an extended period of time (generally 3 weeks or more) following a single injection (Endoh et al., 1992; Jones and Holtzman, 1992; Carroll et al., 2004; Beardsley et al., 2005). This sustained effect may be due to a phenomenon termed biased agonism or ligand-directed signaling, a mechanism by which a ligand can simultaneously act as an agonist and an antagonist at different functions mediated by the same receptor (Urban et al.,

2007; Melief et al., 2011). For KOR antagonists, the duration of action correlates with JNK pathway activation and disrupting JNK activation using inhibitors or mice lacking the JNK-1 isoform blocks the long lasting effects of KOR antagonist (Bruchas et al., 2007b; Melief et al., 2010; Melief et al., 2011). However, the mechanism of JNK activation and JNK-mediated inactivation of the KOR is not known. KOR antagonists block KOR agonist effects and have antidepressant- and anxiolytic-like effects on their own. For example, these antagonists decrease immobility in the FST (Mague et al., 2003; Beardsley et al., 2005; Carr et al., 2010) similar to standard antidepressants (Cryan et al., 2002). Administration of norBNI and JDTic produces anxiolytic-like effects in the EPM and fear-potentiated startle (Knoll et al., 2007), and the irreversible KOR antagonist DIPPA produces anxiolytic-like effects in novelty-induced hypophagia and defensive burying tests in rats (Carr and Lucki, 2010), suggesting that KOR activation is necessary for the acquisition and/or expression of anxiety-like behavior. Anxiolytic-like effects are also observed in Pdyn-deficient mice which show increased center exploration in an open field and spend more time in the lit compartment of a light/dark box (Wittmann et al., 2009), both anxiolytic-like effects. However, other studies indicate existing lines of constitutive KOR-KO mice do not differ from controls in the open field and light/dark box (Simonin et al., 1998; Filliol et al., 2000). During development, gene expression and system functions related to the absent gene may alter to compensate for the gene loss. This is especially relevant in the above case, since the discrepant results are from mice with ablation of different elements of the KOR system knocked out (Pdyn vs. KOR). Dynorphin is a potential ligand for other opioid-receptors (Zhang et al., 1998), and this may underlie compensatory and behavior differences between the two KO strains.

3.4 KOR system and stress-induced behaviors

Stressors including forced swimming (McLaughlin et al., 2003a; Land et al., 2008), footshock (Beardsley et al., 2005; Redila and Chavkin, 2008), and social defeat (McLaughlin et al., 2006b) all activate the KOR system, suggesting it plays an important role in stress effects. In CPP tests, the rewarding effects of cocaine become associated with the environment in which it was paired, thereby causing a preference for this environment during subsequent drug-free exposure. This effect can be extinguished by repeated access to the testing apparatus, and the rapidly reinstated by stress or cocaine priming. Disruption of KOR signaling blocks stress, but not cocaine-primed reinstatement (Carey et al., 2007; Redila and Chavkin, 2008; Aldrich et al., 2009), suggesting the KOR system plays a specific role in stress-induced effects. Stress exposure can also increase the magnitude of cocaine reward, as measured in the CPP test. Potentiation of cocaine-induced CPP following social defeat and forced swim stress is blocked by norBNI and absent in Pdyn and KOR KO mice (McLaughlin et al., 2003a; McLaughlin et al., 2006a; McLaughlin et al., 2006b; Schindler et al., 2010), and activation of KORs mimics the effect of stress (McLaughlin et al., 2006a; Schindler et al., 2010), demonstrating that KOR activation is a necessary and sufficient element of at least certain stress effects on behavior. Although this may seem contradictory to studies described earlier in which KOR agonists blocked effects of drugs of abuse, this is likely due to differences in timing of administration. In those studies KOR agonists were coadministered with the drug of abuse, but in cocaine-induced CPP studies, KOR agonist was injected one hour before cocaine testing. In fact, when the interval between KOR agonist and cocaine was reduced, place aversion was observed (McLaughlin et al., 2006a). Prior activation of the KOR system may decrease hedonic

state and increase the rewarding effects of cocaine (Negus, 2004; Ahmed and Koob, 2005) resulting in potentiation of drug effects (i.e., a larger change due to a lower hedonic baseline) which would not occur with coadministration (Bruchas et al., 2010).

Dynorphin release is also necessary for stress-induced aversion. It is known that mice will develop a CPA to an odorant that was previously paired with stress. The avoidance behavior is abolished by pretreatment with the KOR antagonist norBNI before stress (forced swim or footshock) and absent in Pdyn KO mice (Land et al., 2008), suggesting reduced aversions. Additionally, dynorphin plays a role in stress-induced analgesia, a phenomenon in which stress reduces sensitivity to pain. NorBNI blocks both psychological (Takahashi et al., 1990) and physical stress-induced analgesia (McLaughlin et al., 2003a; McLaughlin et al., 2006a) including CSDS. Both norBNI and Pdyn gene disruption block stress-induced analgesia observed immediately after the first and subsequent days of social defeat (McLaughlin et al., 2006b). During CSDS sessions, rodents display characteristic immobility and social defeat postures, which tend to increase progressively (Miczek et al., 2004). Postures reflecting social defeat are reduced in norBNI treated and Pdyn KO mice, suggesting that KOR blockade produces signs of stress resilience. However, these differences are not apparent proceeding the first day of stress, revealing that KOR signaling is not necessary for initial defeat-induced postures in this paradigm, but instead in the progression of chronic stress. KOR antagonists and KOR system gene disruption also reduce immobility in the FST, but primarily not until the second day (Pliakas et al., 2001; Mague et al., 2003; McLaughlin et al., 2003a; McLaughlin et al., 2006a). Thus KOR-dependent behaviors likely involve distinct neural circuits and may reflect the differences in immediate actions

of KOR activation versus delayed effects such as gene expression changes, which may be especially important in animal models involving initially normal (i.e., non-depressed) subjects.

3.5 KOR system and CRF interactions

As described earlier, CRF is the primary regulator of the stress response; when centrally administered, it can recapitulate many of the behavioral, hormonal and autonomic effects of stress including dynorphin release. CRF stimulates release of dynorphin from spinal cord (Song and Takemori, 1992), hypothalamus (Almeida et al., 1986; Nikolarakis et al., 1986), globus pallidus and striatum (Sirinathsinghji et al., 1989). It also produces increases in KOR phosphorylation—a marker of receptor activation (McLaughlin et al., 2003b)—in components of stress and anxiety circuits including the striatum, DRN, AMY, HIP, VTA, and NAc that are reduced or absent in norBNI pretreated mice and Pdyn KOs (Land et al., 2008). The fact that CRF and KOR agonists produce aversive and anxiogenic-like effects raises the possibility that CRF effects may be mediated by the KOR system. To address this question, Land et al. (2008) examined the effect of KOR blockade on CRF-induced CPAs in mice. In these experiments, central CRF administration induced aversion to the context in which mice were placed following infusion. CRF-induced CPA was abolished with norBNI pretreatment and in Pdyn KO mice (Land et al., 2008), suggesting that CRF receptor activation promotes dynorphin release and subsequent KOR activation to mediate the aversive component of stress. Work I have conducted during my graduate training has also identified important interactions between the CRF and KOR systems in rats using the 5-choice serial reaction time task (5CSRTT), a test of cognitive behavior analogous

to the continuous performance task used to study attention in humans (Beck et al., 1956; Robbins, 2002). CRF dose-dependently disrupted numerous performance in the 5CSRTT, and these deficits were attenuated by systemic administration of JDTic at a dose without effects of its own (see Appendix; Van't Veer et al., 2012). These findings further demonstrate that KOR antagonists can prevent acute CRF-related effects, including those that degrade performance in tasks requiring attention.

CRF induced anxiety-like behavior is also dependent on the KOR system. Mice administered central CRF avoid the open arms of an EPM, an effect that is abolished with prior norBNI treatment or Pdyn gene disruption (Bruchas et al., 2009). The basolateral nucleus of the AMY (BLA) appears critical for this anxiogenic effect, since direct injection of norBNI into this region is sufficient to block CRF-induced decreases in open arm time (Bruchas et al., 2009). This is in agreement with other work demonstrating anxiolytic-like effects of KOR antagonism in the EPM after direct microinfusion into the BLA (Knoll et al., 2011). Nonetheless, KOR phosphorylation is detected in numerous brain structures following CRF treatment (Land et al., 2008), suggesting additional areas of interaction. Anxiety-like behavior is complex and involves a circuit of interconnected brain regions, which all contribute to a behavioral output; as such, it is unlikely that a single brain region represents the only area in which key interactions between the KOR system and CRF occur. In my dissertation, I focused on the VTA and its projection areas as possible sites of stress/CRF and KOR interactions, due to the involvement of this region in responsiveness to stress, its role in states of motivation and emotion, and its expression of CRF-Rs and KORs.

4. Neural circuits

The following section introduces the stress and fear/anxiety circuits. Overlap between these circuits may represent regions where stress may influence fear and anxiety behavior.

4.1 Stress neurocircuitry

Stress of any type activates the HPA axis, a neural circuit within which the medial parvocellular division of the PVN plays a key role (**Fig. 1.1**). These neurons contain CRF and release this peptide from the median eminence of the hypothalamus into the hypophyseal portal system in response to stress (Antoni, 1986). Activation of CRF receptors within the anterior pituitary regulates ACTH release, which acts on the adrenal cortices to promote production and release of glucocorticoids. Many CRF neurons also co-express arginine vasopressin (AVP), which amplifies CRF effects on ACTH release (Gillies et al., 1982; Whitnall et al., 1987). Glucocorticoid actions are mediated by two receptors: glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs). GRs are expressed throughout the brain and are highly expressed in the hypothalamus, olfactory bulb, HIP and cerebral cortex while MRs are more limited in distribution and are predominantly found in areas involved in emotion, memory, and behavior such as the septum, HIP, and PFC (Fuxe et al., 1985; Reul and de Kloet, 1985; Ahima and Harlan, 1990; Cintra et al., 1994; Morimoto et al., 1996; Viengchareun et al., 2007). GRs and MRs regulate hormonal, autonomic and behavior responses to stress via their widespread expression (Munck et al., 1984), and trigger a negative feedback loop that terminates HPA axis activation. Glucocorticoids act within the PVN to suppress CRF and AVP mRNA levels and, as a result, inhibit ACTH release (Swanson and Simmons,

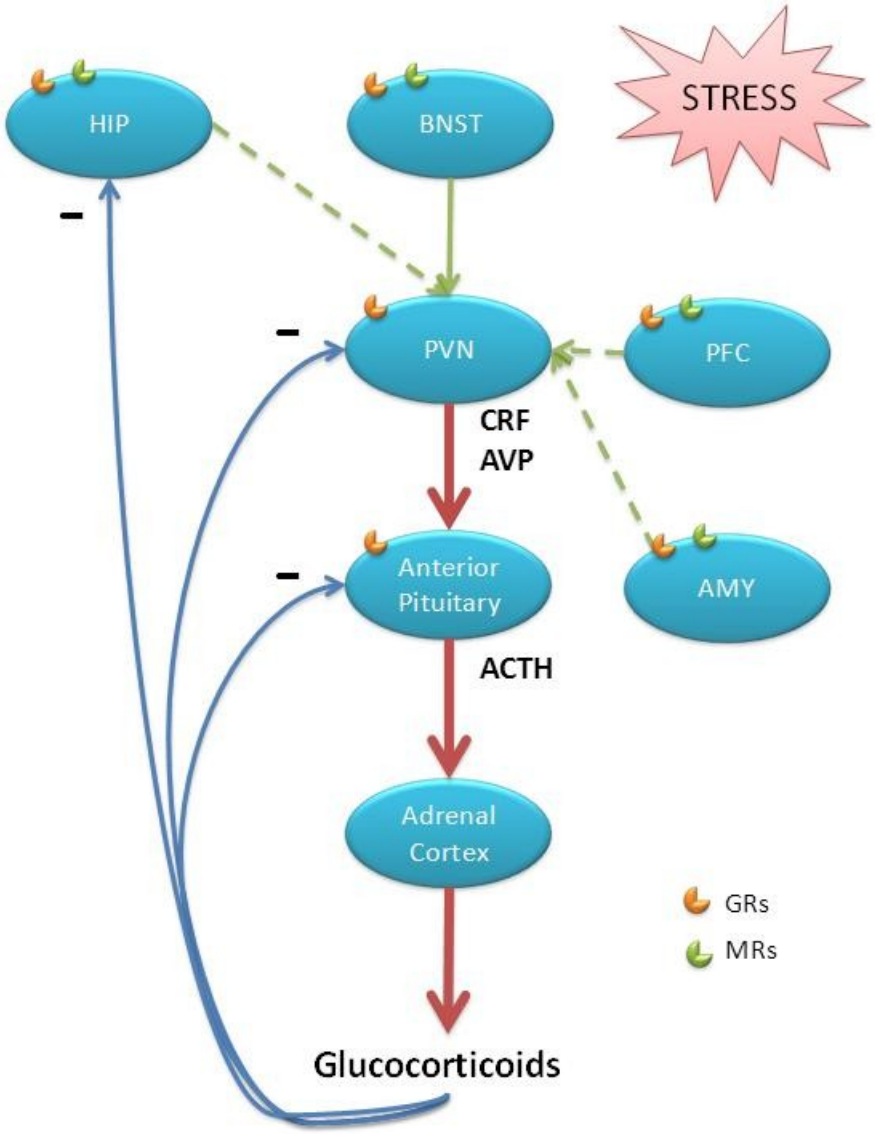
1989; Autelitano et al., 1990). It has been proposed that other negative feedback circuits controlling gene expression and hormone release in the PVN may exist, considering the diverse expression of GRs and MRs. Indeed, lesions of the HIP result in elevated glucocorticoids and elevated levels of CRF and AVP, suggesting disruption of negative feedback regulation (Herman et al., 1989).

HPA axis regulation is achieved through actions integrated within the PVN. Afferents from cicumventricular organs, brainstem nuclei, and hypothalamic-basal forebrain systems can directly activate PVN neurons (Ziegler and Herman, 2002) and relay information on the state of the body such as cardiovascular tone, blood oxygenation, arousal and osmotic state. In particular, the BNST sends projections from multiple subregions (Dong et al., 2001b; Ziegler and Herman, 2002; Dong and Swanson, 2004), suggesting that this region plays a crucial role in regulation of PVN activity. The BNST also integrates information from other stress-responsive brain regions. Brain mapping studies of immediate-early gene (IEG) expression have been used to understand the circuits mediating the positive regulation of the stress response. Stress leads to generalized activation of the IEG c-fos in numerous brain regions including the HIP, NAc, BNST, AMY, PVN, DRN, CPu, and VTA (Lopez et al., 1999). The AMY, HIP and PFC can regulate the stress response (Herman et al., 2005) despite having sparse or no direct connections with the PVN (Gray et al., 1989; Cullinan et al., 1993; Ziegler and Herman, 2002). These regions do send projections to the BNST (Cullinan et al., 1993; Canteras et al., 1995; Crane et al., 2003), suggesting this region may represent a relay station where limbic information feeds in and is then passed to the PVN (Cullinan et al., 1993; Herman et al., 2005). Likewise, other regions with direct input to the PVN relay

information from afferent connections. For example, observed central AMY actions on HPA axis function (Beaulieu et al., 1987; Xu et al., 1999) likely rely on indirect projections to the PVN via the nucleus of the solitary tract (Schwaber et al., 1982; Ziegler and Herman, 2002). Interestingly, the major limbic components of the hormonal stress response have also been implicated in fear and anxiety neurocircuitry.

Figure 1.1 HPA axis and neuronal inputs. Stress causes the release of CRF and AVP from parvocellular neurons in the PVN that project to the anterior pituitary. ACTH secretion then leads to glucocorticoid synthesis and release from the adrenal cortex. Glucocorticoid actions are mediated by GRs and MRs throughout the brain and periphery. Glucocorticoids activate negative feedback loops within the PVN, pituitary and HIP denoted with minus signs in the illustration. Neuronal inputs from the HIP, BNST, PFC and AMY regulate HPA axis (red arrows) activity. Dashed lines represent indirect connections to the PVN. ACTH, adrenocorticotropic hormone; AMY, amygdala; AVP, arginine vasopressin; BNST, bed nucleus of the stria terminalis; CRF, corticotropin-releasing factor; GR, glucocorticoid receptor; HIP, hippocampus; HPA, hypothalamic-pituitary-adrenal; MR, mineralocorticoid receptor; PFC, prefrontal cortex; PVN, paraventricular nucleus of the hypothalamus

Figure 1.1 (Continued) Hypothalamic-pituitary-adrenal (HPA) axis and neuronal inputs



4.2 Fear and anxiety neurocircuitry

There is considerable overlap in the stress circuits described above and those involved in fear and anxiety, suggesting that stress and fear responses are related and can affect one another (Shin and Liberzon, 2010). The AMY is the brain region most often considered to be at the center of the fear response. Over 50 years ago, physicians surgically treating epileptic patients reported that electrical stimulation of the AMY produces feeling of fear and anxiety (Chapman et al., 1954). Decades later, the significance of the AMY became clearer as researchers focused on Pavlovian conditioning. Much preclinical work has elucidated AMY cellular and molecular mechanisms in fear as reviewed elsewhere (Davis, 1997; Davis and Shi, 2000). Although the AMY is clearly involved in the expression of fear and anxiety behaviors, it is embedded within a circuit of highly interconnected brain structures that are known to be involved in processes that reflect motivation and emotion. It is increasingly evident that structures with amygdalar afferent and/or efferent projections contribute to normal and pathologic anxiety. Although the AMY is a macrostructure that includes numerous sub-regions, most research on fear and anxiety has focused on two: the BLA, and the central nucleus of the AMY (CeA) (Davis, 2002). These regions have different cell types and projection patterns (Krettek and Price, 1978; Carlsen and Heimer, 1988; Sah et al., 2003). The BLA contains predominantly glutamatergic pyramidal neurons and receives input from the cortex, HIP and thalamus. This integrated information may then be passed on to efferent structures including the striatum, HIP, PFC, CeA and BNST. The CeA contains predominantly medium spiny (GABA-containing) neurons and is a major output center that projects to numerous brain regions involved in specific components of fear and anxiety. A deeper understanding of how these interconnected

regions function in isolation as well as in circuits may enable new insights into the neurobiology of stress and anxiety responses as well as the pathophysiology of psychiatric disorders. In the following section I focus on the role of the VTA in regulating the activity of AMY circuits.

4.2.1 Ventral tegmental area

Although not implicated in classical theories of depressive illness, the mesocorticolimbic system may also play an important role in affective behavior (Nestler and Carlezon, 2006). The VTA is the origin of neurons of the mesocorticolimbic DA system which project to the NAc, HIP, AMY, PFC and BNST. Historically, the VTA and its dopaminergic projections have been studied primarily in the context of motivation and reward (Wise and Bozarth, 1987). However, accumulating work has led to greater recognition of the role of this system in aversion as well (Salamone, 1994; Pezze and Feldon, 2004; Carlezon and Thomas, 2009). Aversive stimuli can increase DA neuron population activity (Valenti et al., 2011) and activate the mesocorticolimbic system resulting in postsynaptic DA release (Thierry et al., 1976; Abercrombie et al., 1989; Imperato et al., 1993; Piazza and Le Moal, 1998; Pascucci et al., 2007) that may promote or antagonize stress effects on behavior.

4.2.1.1 Role of DA

Early studies investigating the effect of systemic dopaminergic drugs on fear conditioning found that, in general, DA receptor agonists potentiate fear conditioning (Tsuchiya et al., 1996; Borowski and Kokkinidis, 1998; Pezze et al., 2002), whereas antagonists have the opposite effect (Hijzen et al., 1995; Inoue et al., 1996; Greba and

Kokkinidis, 2000). These effects were likely due to activation of receptors within and postsynaptic to dopaminergic midbrain neurons, the main source of DA in the mammalian CNS. Daily VTA stimulation alone is sufficient to produce fear-like behavior in cats (Stevens and Livermore, 1978) and aversive stimuli such as footshock increase DA metabolites in the VTA (Deutch et al., 1985), which may indicate increased DA release. Interestingly, these same effects are seen following the presentation of auditory cues previously paired with footshock, demonstrating that the VTA responds similarly to unconditioned and conditioned stimuli. When VTA function is disrupted by axon-sparing lesions or inhibition of DA neuron activity, fear-potentiated startle is reduced without affecting baseline startle (Borowski and Kokkinidis, 1996), suggesting specific effects of VTA activation in response to fear-eliciting stimuli. In rabbits, the firing rate of dopaminergic VTA neurons changes depending on whether a conditioned stimulus (CS) previously paired with a shock or a neutral stimulus was presented, with the majority of neurons showing greater activity to CS-paired compared to unpaired presentations (Guarraci and Kapp, 1999). These changes in firing rate may transfer to subsequent changes in excitability and function of innervated target regions. Together, these data indicate that DA release and activation of DA-receptor expressing neurons contribute to the expression of fear- and anxiety-like behavior in rodents. However, how these changes in DA release can lead to anxiety-like behavior is not fully understood.

There is some evidence that the VTA to NAc projection is involved in the acquisition and expression of fear conditioning. Various stressors have been shown to increase DA metabolites or release in the NAc (Abercrombie et al., 1989; Sorg and Kalivas, 1991; Imperato et al., 1993; Inoue et al., 1996). DA levels also rise in response to a CS

previously paired with an aversive unconditioned stimulus (US) (Young et al., 1993; Wilkinson et al., 1998; Pezze et al., 2002). During conditioning trials, DA release is higher during CS-US pairings than when the US is presented alone suggesting the NAc is important for CS-US associations (Pezze and Feldon, 2004). Further, during test trials where the CS no longer predicts the US, DA release decreases (Wilkinson et al., 1998). Thus NAc DA likely encodes the salience of the CS (Horvitz, 2000; Pezze and Feldon, 2004).

Although it may seem paradoxical that aversive and fear-eliciting stimuli lead to similar increases in NAc DA as observed following rewarding stimuli (including drugs of abuse, food, and sexual behavior; Wise and Rompre, 1989), the perception of reward versus aversion may also rely on concurrent activation of other circuits that provide input to the NAc, thereby affecting the ways in which neural signals are integrated and gated (West et al., 2003). For example, the ability for intra-NAc CRF to induce anxiety-like behavior may rely on the balance between DA and acetylcholine release in this region (Chen et al., 2012). Importantly, distinct DA pathways are recognized for reward, aversion, and salience (Lammel et al., 2011). Whereas DA release within subregions of the NAc may encode salience, aversive responses may be encoded, at least in part, by mesocortical neurons. The PFC is an important site in modulating conditioned fear responses, although the precise mechanism is unclear (Pezze and Feldon, 2004). Both stimulation and blockade of DA within the PFC have been shown to reduce the expression of conditioned fear which may reflect an inverted U-shaped relationship between fear expression and DA activity (Abercrombie et al., 1989; Imperato et al., 1992; Sorg and Kalivas, 1993; Dazzi et al., 2001; Feenstra et al., 2001; Bassareo et al., 2002; Pezze et

al., 2003). Depletion of DA within the PFC also produces negative effects including impairment of extinction learning and anxiogenic-like effects (Espejo, 1997; Fernandez Espejo, 2003). Conversely, neuropeptide S increases DA in the PFC (Si et al., 2010), an effect that may underlie its ability to reduce stress-related anxiety. Together, these data indicate that manipulations in DA may affect fear and anxiety-like behavior in region specific ways.

A small number of studies in human subjects have supported preclinical findings and demonstrated a role for the NAc in anxiety. In a case report of a patient undergoing deep brain stimulation (DBS) surgery for obsessive compulsive disorder, macrostimulation of the ventral NAc induced feelings of fear and panic, which were coincident with stimulation (Shapira et al., 2006). This effect is in contrast to the use of NAc DBS for the treatment of anxiety and obsessive-compulsive disorder (OCD). Indeed, this patient continued to receive DBS as treatment at contacts separate from those which induced “panic” (Shapira et al., 2006). In other studies, anxiety and OCD symptoms were significantly relieved in 3 patients implanted with NAc DBS stimulators (Sturm et al., 2003), and depression and anxiety rating improved in patients with treatment-resistant depression comorbid with anxiety (Bewernick et al., 2010; Bewernick et al., 2012). DBS at high frequencies is thought to inhibit activity in the targeted region and produce effects similar to lesion (Blond et al., 1992; Benazzouz and Hallett, 2000), but is also capable of increasing neuronal activity at the site of stimulation (Montgomery and Gale, 2008). Thus positive effects of DBS may be due to functional inactivation of NAc while inductions of fear may result from activation. Since DBS may cause antidromic activation (Nowak and Bullier, 1998), these differences may

also rely on excitatory and inhibitory inputs to the site of stimulation which are heterogeneous in the NAc (Heimer et al., 1997). Regardless of mechanism, these studies identify the NAc and its inputs as key components in anxiety circuitry in humans and support the use of rodent models to investigate the mechanisms of fear and anxiety.

4.3. KOR effects in the mesocorticolimbic system

Insight into how the mesocorticolimbic system is involved in anxiety may come from studies of depressive-like behavior. In humans, depression is highly comorbid with anxiety disorders with over half of depressed patients meeting the diagnostic criteria for an anxiety disorder (Kaufman and Charney, 2000; Kessler, 2000). Stress has been shown to cause behaviors characteristic of depression such as anhedonia, behavioral despair, and dysphoria in rats (Moreau et al., 1992; Pliakas et al., 2001; Land et al., 2008) that are mimicked by elevating CREB levels in the NAc using viral-mediated gene transfer (Pliakas et al., 2001; Barrot et al., 2002). In contrast, decreasing CREB activity in the NAc through expression of a dominant-negative CREB virus leads to antidepressant-like effects in rodents (Pliakas et al., 2001; Newton et al., 2002). Notably these changes in behavior due to increases or decreases in CREB activity were shown to be mediated largely by CREB-induced changes in dynorphin expression. Dynorphin has been shown to be a target of CREB induced gene expression *in vitro* (Douglass et al., 1994; Cole et al., 1995; Turgeon et al., 1997) and manipulating CREB levels changes dynorphin expression *in vivo* (Carlezon et al., 1998; Pliakas et al., 2001). Administration of norBNI attenuated the behavioral effects of elevated CREB levels within the NAc (Carlezon et al., 1998; Pliakas et al., 2001), whereas blockade of

endogenous dynorphin actions through direct injection of norBNI into the NAc was sufficient to produce antidepressant-like effects (Newton et al., 2002). It is postulated that some features of depression are the result of dynorphin control of mesocorticolimbic DA function, either by actions at KORs on VTA cell bodies or terminals that project to the NAc (Nestler and Carlezon, 2006). Given the high comorbidity of depressive and anxiety disorders, KOR signaling and control of DA function may underlie the pathophysiology of both. In my dissertation project, I used molecular and genetic manipulations to directly determine if stress/CRF and the KOR system interact in the mesocorticolimbic system in models of anxiety-like behavior.

In order to probe the relationship between KORs and stress-induced behaviors, I subjected mice to stress and then measured the acoustic startle response. This response is a reflectory reaction present in all mammals that is thought to facilitate escape from sudden stimuli, and it can be modulated by emotional state such that fear and anxiety increase startle whereas pleasure decreases it (Davis, 1979; Schmid et al., 1995). The startle reflex has many advantages as a metric, including the fact that it can be used in species ranging from humans to mice. Elevations in the startle reflex are a hallmark sign of serious anxiety disorders such as PTSD, and may reflect clinical diagnostic criteria such as hyperarousal and hypervigilance (American Psychiatric Association, 2000). I used infusions of CRF or footshocks as aversive stimuli as described below, and used acoustic startle as my main behavioral output. (I did, however, use a wide variety of other behavioral procedures when characterizing novel lines of mutant mice, as will be described in Chapter 4 of this thesis.)

5. Main behavioral paradigms

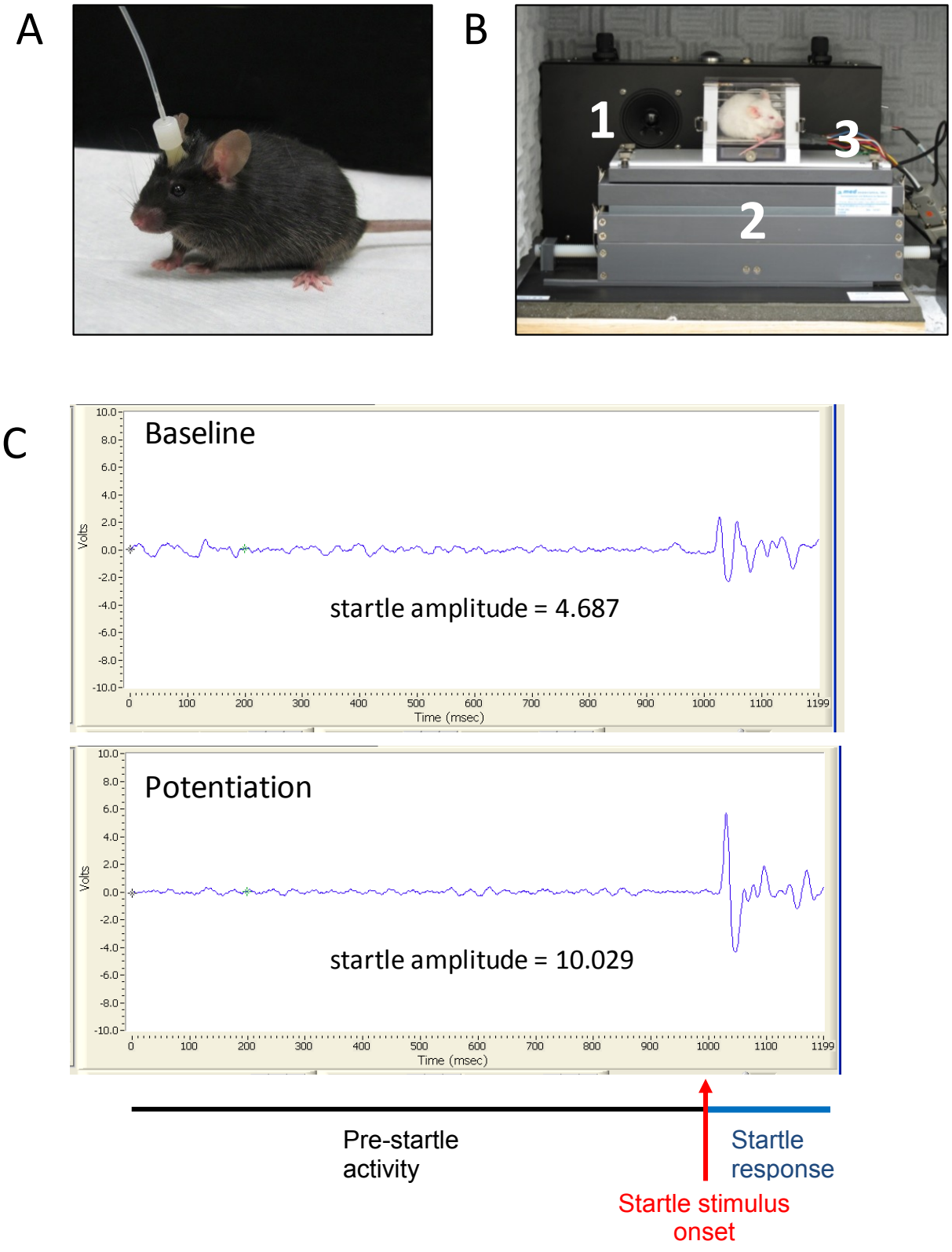
5.1 CRF-enhanced startle

Stress-like states in rodents can be achieved through central administration of CRF. One effect of CRF is enhancement of the startle response. This CRF-enhanced startle is a reliable measure with face, predictive, and construct validity. The startle response is a robust reflex in response to an intense acoustic, tactile, or vestibular stimulus (Yeomans et al., 2002). In rodents this response is measured by a full body flinch. The magnitude of this response can be increased when fear-inducing stimuli are presented at the time of the startle stimuli or when anxiogenic compounds, such as CRF are administered (Swerdlow et al., 1986; Davis, 1993). It is believed that CRF induces a central state of anxiety in rodents since fear-conditioning or administration of compounds known to be anxiogenic in humans, such as yohimbine (an α2-adrenergic receptor antagonist), result in similar increases in startle amplitude (Morgan et al., 1993; Grillon and Davis, 1997). Furthermore, the CRF-enhanced startle paradigm shows predictive validity since compounds that reduce anxiety in humans, such as benzodiazepines, reduce the CRF-enhanced startle response in rodents (Swerdlow et al., 1986). It is centrally mediated and not dependent on corticosterone release from the adrenal glands, since adrenalectomized rats still show enhanced startle (Lee et al., 1994). In the CRF-enhanced startle paradigm, CRF is administered to rodents via cannulae directed at the lateral ventricle (**Fig. 1.2A**), since the CRF peptide does not effectively cross the blood brain barrier. Startle reactivity is then measured using a specialized automated system comprising a speaker capable of delivering startle stimuli (sudden bursts of white noise), a holder with a metal rod floor that positions the mouse directly in front of the speaker, and a load cell upon which the chamber is placed that

can quantify the force of the startle response (**Fig. 1.2B**; white mouse shown for clarity). Startle data are measured as the peak to peak response amplitude in the first 100 msec following startle stimuli onset. Sample startle data showing theoretical baseline (**Fig. 1.2C**) and potentiated (**Fig. 1.2D**) startle responses illustrate the increase in (measured in arbitrary startle units) seen following central CRF treatment compared to baseline or vehicle infusion.

Figure 1.2 Startle apparatus. ***A***. In the CRF-enhanced startle paradigm, mice are infused with vehicle (aCSF) or CRF via an intracerebroventricular cannula immediately before testing. ***B.*** The startle apparatus includes a speaker (1) located behind the mouse holder that delivers startle-stimuli consisting of bursts of white noise. The startle response is quantified by a load cell (2) beneath the holding cage which converts the force of the cage displacement into arbitrary startle units. In the footshock-potentiated startle paradigm, mice are not infused with CRF and are instead given mild footshocks delivered via a wiring harness connected to floor rods (3) to induce a fear-like state. ***C.*** Hypothetical output data of startle responding showing baseline data before aversive experimental manipulation and the potentiated response expected following central CRF infusion or footshock.

Figure 1.2 (Continued) Startle apparatus



5.2 Footshock-potentiated startle

Fear-like states can be achieved through brief presentation of mild shock. The startle response is enhanced immediately after shock, delivered to the feet of rodents (Boulis and Davis, 1989; Davis, 1989; Hitchcock et al., 1989; Sasaki and Hanamoto, 2007) or arm of humans (Greenwald et al., 1998). This behavior was originally thought to reflect unlearned fear and named “shock sensitization” (Davis, 1989). Subsequent work, however, has shown that this increase in startle is due to rapid contextual conditioning (McNish et al., 1997; Richardson, 2000; Risbrough et al., 2009) and is thus a model of learned fear. Footshock has been shown to induce CRF release (Wang et al., 2005), while disrupting CRF receptor activation can block the behavioral effects of footshock (Ho et al., 2001; Bakshi et al., 2002; Le et al., 2002). Footshock-potentiated startle is abolished in CRF-R1 KO mice and reduced in CRF-R2 KOs (Risbrough et al., 2009). Since footshock-enhanced startle also depends on CRF receptor activation, I chose this paradigm as a model of learned fear to complement my CRF-enhanced startle studies. These studies involve the same basic apparatus as is used for the CRF-enhanced startle studies; in this case, however, scrambled footshock is delivered through metal rods at the bottom of holding chamber (**Fig. 1.2B**).

6. Summary

My working hypothesis is that KORs within the mesocorticolimbic circuit play an important role in regulating anxiety-like behavior. In my first set of studies to address this hypothesis, I tested the effects of systemic KOR antagonism in CRF-enhanced and footshock-potentiated startle. This work is described in Chapter 2.

Chapter 2

Antagonism of kappa-opioid receptors reduces corticotropin-releasing factor-induced effects on startle

Ashlee Van't Veer[1], F. Ivy Carroll[2], William A. Carlezon, Jr.[1]

[1]Department of Psychiatry, Harvard Medical School, McLean Hospital, Belmont, MA 02478; [2]Research Triangle Institute, Research Triangle Park, NC

Author contributions: AVV and WAC designed the experiments. AVV conducted the experiments and analyzed the data. FIC synthesized and provided JDTic.

Abstract

Rodent models used in research on psychiatric illness have implicated the KOR system in anxiety-like behavior. KOR antagonists reduce anxiety in models of learned and unlearned fear, and block the effects of CRF, a key regulator of the stress response. The present studies further characterize interactions of KOR-CRF systems by determining if KOR antagonists can affect increases in startle reactivity produced by central infusion of CRF. Mice were administered intraperitoneal (IP) injections of the selective and long-lasting KOR antagonist JDTic (10 or 30 mg/kg) immediately following surgery to implant a guide cannula directed at the lateral ventricle for delivery of CRF. One week later, they were tested for startle responsiveness following intracerebroventricular (ICV) administration of CRF (1.0 µg). JDTic dose-dependently attenuated CRF-induced increases in the acoustic startle response without affecting baseline startle reactivity. The persistent ability of JDTic to disrupt KOR function was confirmed using the tail-flick assay. JDTic-induced reductions in startle were accompanied by decreases in c-Fos immunoreactivity in the VTA and dorsal HIP. Double-labeling experiments in the VTA revealed that JDTic significantly reduced c-Fos cell counts in dopaminergic neurons compared to CRF treated controls. Stress is known to increase dopaminergic transmission, and these results indicate that KORs may be involved in regulation of this process. Further, these data indicate KOR antagonists may be particularly useful for treating anxiety disorders in which CRF systems are dysregulated, such as PTSD.

Introduction

The stress response is an adaptive process that preserves homeostasis and enhances the chances of survival. However, prolonged or uncontrolled stress can precipitate psychiatric illness, including anxiety and depressive disorders (Keane et al., 2006; Keller et al., 2007; Kessler et al., 2010). CRF is a brain peptide often considered the principal regulator of the stress response (Spiess et al., 1981; Majzoub, 2006). Its effects are mediated through two cognate receptors (CRF-1 and CRF-2) that are found throughout the brain including regions implicated in stress and anxiety including the AMY, VTA, and NAc (De Souza et al., 1985; Millan et al., 1986; Van Pett et al., 2000; Dautzenberg and Hauger, 2002). Central infusions of CRF induce anxiety-like behavior in laboratory animals including potentiation of the acoustic startle response (Swerdlow et al., 1986; Liang et al., 1992), a hallmark symptom of PTSD (American Psychiatric Association, 2000). Several studies have reported elevated levels of cerebrospinal fluid CRF in PTSD patients (Bremner et al., 1997a; Sautter et al., 2003; de Kloet et al., 2008), suggesting that blocking CRF receptor actions may be useful therapeutically. Indeed, CRF antagonists decrease anxiety and fear behavior in non-human primates (Habib et al., 2000) and patients with depression (Zobel et al., 2000). However, development of such drugs for clinical use has been unsuccessful due to setbacks such as poor efficacy and unwanted side effects (Zorrilla and Koob, 2010). Identification of neural processes that occur downstream of CRF actions may enable the development of improved medications to treat or prevent stress-related illnesses while having fewer side effects.

KORs are selectively activated by the endogenous opioid dynorphin (Chavkin et al., 1982). We have shown previously that systemic KOR antagonism produces anxiolytic-like effects in tests of learned and unlearned fear (Knoll et al., 2007). Further, KOR antagonist treatment and KOR system KO mice have reduced stress-induced behaviors such as analgesia, immobility in the FST, and potentiation of cocaine-induced CPP (Takahashi et al., 1990; Menendez et al., 1993a; McLaughlin et al., 2003a). KOR activation has also been suggested to mediate actions of CRF on behavior. Indeed, CRF increases phosphorylation (i.e., activation) of KORs in the brain (Land et al., 2008; Bruchas et al., 2009). Inhibiting KOR action through receptor blockade or using dynorphin KO mice reduces effects of CRF on open arm exploration (Bruchas et al., 2009) and abolished CRF-induced place aversions (Land et al., 2008). Evidence suggests that the stress-related effects of KOR activation are downstream of CRF activation since U50,488-induced place aversions—which mimic CRF effects—are not blocked by CRF-R antagonism (Land et al., 2008). If so, it is conceivable that disruption of KOR function could mitigate the aversive or maladaptive effects of stress without affecting any beneficial (i.e., pro-adaptive or homeostatic) effects.

The brain circuits involved in KOR regulation of stress effects are not well understood. Central administration of CRF leads to KOR activation in the NAc, BLA, HIP and DRN (Land et al., 2008). Elevated KOR system function within the NAc and HIP has been implicated in depressive-like behavior in rodents. For example, stress produces upregulation of CREB (which regulates dynorphin) (Pliakas et al., 2001; Newton et al., 2002; Shirayama et al., 2004) and dynorphin expression (Shirayama et al., 2004; Chartoff et al., 2009) within these regions. In contrast, KOR activation within the AMY

has been implicated in anxiety-like behavior. For example, microinfusions of KOR antagonists into the AMY produces anxiolytic-like effects: rodents show reduced conditioned fear as measured by fear-potentiated startle, increase open arm exploration in the elevated plus maze (Knoll et al., 2011), reduced swim stress and CRF-induced anxiety-like behavior (Bruchas et al., 2009), and reductions in the stress-related effects of nicotine (Smith et al., 2012). Considering the high comorbidity of depressive and anxiety disorders in humans (Kaufman and Charney, 2000; Kessler et al., 2003), and the fact that all of these regions receive input from the VTA (Swanson, 1982), there may be substantial overlap in the brain circuits that regulate depressive- and anxiety-like effects.

The present studies were designed to further characterize how interactions among KORs, dynorphin, and CRF regulate behavior. We quantified anxiety-like behavior in mice using the acoustic startle response. Increases in startle—which likely reflect hyperarousal or hypervigilance—are seen in people with anxiety disorders such as PTSD (American Psychiatric Association, 2000) and are produced in rodents by central infusion of CRF (Liang et al., 1992). CRF-enhanced startle is generally considered to reflect an anxiety behavior in rodents since fear conditioning or administration of compounds known to be anxiogenic in humans, such as yohimbine (an α2-adrenergic receptor antagonist), produce similar increases in startle amplitude (Morgan et al., 1993; Grillon and Davis, 1997). Furthermore, reductions of CRF-induced increases in startle may indicate an anxiolytic effect, since medications that reduce anxiety in humans (e.g., benzodiazepines) reduce the CRF-enhanced startle response in rodents (Swerdlow et al., 1986; Abduljawad et al., 2001). In the present studies, mice were given a single

injection of the long-acting KOR antagonist JDTic (Carroll et al., 2004) before CRF-enhanced startle testing. Mice were then tested in the warm water tail-flick assay to ensure KOR blockade, as demonstrated by the ability of JDTic to block the analgesic effects of a KOR agonist (McLaughlin et al., 2003a; Van't Veer et al., 2012). Finally, c-Fos immunohistochemistry was used to provide an index of neuronal activation (Kovacs, 1998) to probe the effects of JDTic on CRF-induced changes in the activity of stress-related brain circuits.

Materials and Methods

Mice: Experiments were performed in 8-10-week-old male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME). Mice were individually housed upon arrival and allowed to acclimate to the animal care facility for one week prior to surgery. Mice were maintained on a 12:12-h light-dark cycle (0700h lights on) with *ad libitum* food and water available except during testing. Experiments were conducted during the light phase of the daily cycle to avoid startle ceiling effects (Chabot and Taylor, 1992). Experimental protocols were approved by the Institutional Animal Care and Use Committee of McLean Hospital and in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Academies Press, Washington D.C., USA, 2011).

Drugs: (3R)-7-Hydroxy-N-[(2S)-1-[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethylpiperidin-1-yl]-3-methylbutan-2-yl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (JDTic) was synthesized at Research Triangle Institute (Research Triangle Park, NC). *Trans*-(±)-3,4-Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide hydrochloride

(U50,488) was purchased from Sigma-Aldrich (St. Louis, MO). Rat/human CRF was purchased from American Peptide (Sunnyvale, CA). JDTic and U50,488 were dissolved in 0.9% saline and administered by intraperitoneal (IP) injection at 10 mL/kg. The dose of U50,488 was based on its salt form. CRF was dissolved in artificial cerebrospinal fluid (aCSF, Harvard Apparatus, Holliston, MA) and infused in a volume of 1.0 µL.

Stereotaxic surgery: Mice were anesthetized with ketamine/xylazine (100 mg/kg, 12.5 mg/kg, IP) and placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA) with zygoma ear cups in order to prevent damage to the ear drums. For each mouse, a stainless steel guide cannula (26-gauge, Plastics One, Roanoke, VA) with a dummy stylet extending 1.5 mm beyond the end was lowered into the right lateral ventricle at the following coordinates, relative to bregma: anteroposterior = -0.2 mm, mediolateral = 1.0 mm, and lowered -2.4 mm ventral to the skull (Paxinos and Franklin, 2001). Non-acrylic cement permanently secured the cannula to the skull. The mice were allowed to recover for 5-7 days before testing.

Startle testing: Acoustic startle data were collected by measuring the amplitude of the startle reflex in response to white noise bursts of various intensities using the Med Associates Inc. (St. Albans, VT) Startle Reflex System and Advanced Startle software program. Mice were placed into 8.5 x 7 x 7 cm Plexiglas holders with steel rod floor bars attached to a load cell platform contained within a 40 x 64 x 42 cm sound-attenuating chamber. The load cell transduces movement into an electrical output that is amplified and digitized into arbitrary units by an analog-to-digital converter interfaced to a computer. Startle amplitude was defined as the maximum peak-to-peak voltage

occurring within the first 100 ms after the onset of the startle stimulus. An audio stimulator generated 50 msec startle stimuli (1-32 kHz white noise, 1 msec rise-decay) that were delivered through high-frequency speakers located 4 cm behind the cages. The intensities of the startle stimuli were calibrated before use using customized software. All tests were conducted in darkness.

Following recovery from surgery, mice were given a habituation session to acclimate them to the testing chamber and match them into groups with equivalent baseline startle. The habituation session consisted of a 5 min acclimation period followed by startle stimuli at 3 decibel (dB) levels (20 each at 80, 90 and 100 dB) presented in a pseudo-random order with an inter-stimulus interval (ISI) of 30 sec. Two days later they were given ICV infusions of vehicle (aCSF) or CRF. The dummy stylet was removed and replaced with a 33-gauge infusion stylet (Plastics One) attached to a Hamilton microsyringe (10 µl) by polyethylene tubing. ICV infusions (1.0 µL volume) of either vehicle (aCSF) or CRF (0.25, 0.5 or 1.0 µg) were performed over a 2-min period at a rate of 0.5 µL/min, with an additional 2 min of diffusion time before the infusion stylet was removed and the dummy stylet was replaced. During the infusion mice were placed in clean and empty mouse cages divided in half and were free to move. After the infusion, mice were immediately placed in the startle chambers for a 5 min acclimation period followed by a 99-min startle test of 198 startle stimuli (80, 90, or 100 dB presented in a pseudo-random order with an ISI of 30 sec). Mice used to examine the effects of a KOR antagonist on CRF-enhanced startle received JDTic (10 or 30 mg/kg, IP) or vehicle (0.9% saline) immediately following surgery, and 7 d later were tested as above after administration of 1.0 µg CRF (ICV).

Warm water tail-flick assay: The tail-flick assay was used to assess KOR-mediated analgesia (Vaught and Takemori, 1979; McLaughlin et al., 2003a). The test was conducted 24 hr following the final startle test session. Mice were scruffed with their tail free and back supported and approximately 1 cm of their tail was submerged into a 52 ± 1°C warm water bath. The latency for mice to withdraw their tail from the water was timed with a stopwatch. A maximum time of 15 sec was imposed to prevent damage to the submerged tail. After taking baseline latencies, mice were treated with the KOR agonist U50,488 (15 mg/kg, IP), and withdrawal latencies were assessed 30 min later.

Histological verification: Mice were overdosed with pentobarbital (130 mg/kg, IP) and transcardially perfused with 0.9% saline (20 mL) followed by 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS) (60 mL). Brains were removed and postfixed overnight in 4% paraformaldehyde, and then cryoprotected in 30% sucrose/0.1M PBS for 24 h. Brains were sectioned at 30 µm on a freezing microtome and collected in 0.1M phosphate buffer. Sections were mounted on gelatin-coated slides (Fisher Scientific, Pittsburg, PA) and stained with 0.1% cresyl violet. Mice with incorrect placements (e.g., if the tip of the cannula was found to be embedded in brain tissue adjacent to the lateral ventricle, rather than being located within the lateral ventricle itself) or signs of infection were excluded from analysis.

c-Fos immunohistochemistry: Separate cohorts of mice were pretreated with an effective dose of JDTic (30 mg/kg, IP) or vehicle during surgery as described above and allowed to recover for 3 days. Mice were then given mock ICV infusions on four consecutive days to habituate them to the experimental procedure and reduce handling-

induced c-Fos expression. The following day at test time, mice received CRF (1.0 µg) or aCSF vehicle and were placed back in their home cage for 120 minutes and then sacrificed and perfused. Mouse brain sections (40 µm) were first incubated for 30 min in 0.3% H_2O_2, and then in a blocking solution of 5% normal goat serum (NGS; Vector Laboratories, Inc., Burlingame, CA) for 1 h. Sections were then incubated overnight with anti-c-Fos (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 5% NGS. The following morning, sections were first incubated for 1 hr in biotinylated anti-rabbit IgG (1:1,000; Vector Laboratories), and then in avidin-biotin complex (ABC) for 1 hr (ABC Elite Kit, Vector Laboratories). C-Fos immunoreactivity was visualized with 3,3'-diaminobenzidine (DAB). Photomicrographs of regions of interest were obtained at 40x on a Zeiss Axio Scope (Carl Zeiss Inc., Thornwood, NY) using OpenLab software (PerkinElmer, Waltham, MA). The number of labeled cells for each photomicrograph was counted using ImageJ software (NIH, Bethesda, MD) and the counts from 3 non-serial sections per region were averaged for each mouse

For double-labeling with anti-tyrosine hydroxylase (TH), sections were labeled for c-Fos as described. Sections were then incubated in primary antibody to TH (1:10,000; Millipore, Billerica, MA) in 5% NGS overnight followed by biotinylated anti-rabbit IgG (1:1,000) and ABC for 1 hour each. TH immunoreactivity was visualized with nickel-enhanced DAB. C-Fos, TH and double labeled cells were counted from bilateral 40X images of the VTA from 3 non-serial sections using Stereo Investigator software (MBF Bioscience, Williston, VT).

Data analysis: For the CRF dose response, 2-way (CRF x dB level) analysis of variance (ANOVA) with repeated measures (dB level) were used to compare the effects of increasing doses of CRF to aCSF vehicle. The effect of JDTic on CRF-enhanced startle was analyzed with a 3-way (JDTic x CRF x dB level) ANOVA with repeated measures (dB level). For the tail-flick assay, a 3-way (JDTic x CRF x U50,488) ANOVA with repeated measures (U50,488) was used to examine whether JDTic affected U50,488-induced antinociception. Significant interactions in the ANOVAs were further analyzed using Bonferroni *post hoc* tests. Counts from c-Fos and TH double-labeling were analyzed by t-test. Data are graphed as mean plus standard error of the mean (SEM).

Results

A total of 7 mice were excluded from analysis after histological assessment which indicated the tip of the cannula was embedded in brain tissue instead of terminating in the lateral ventricle. All other mice had correct placements (**Fig. 2.1**).

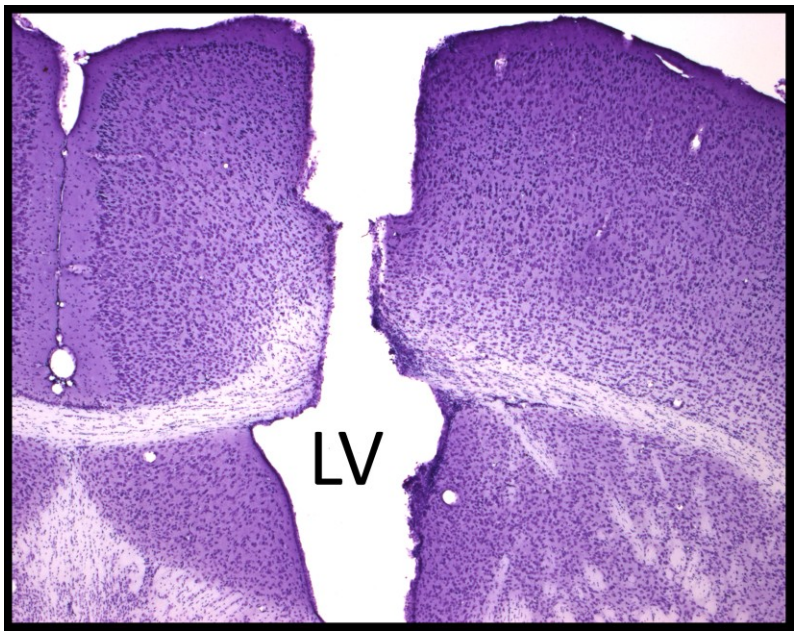
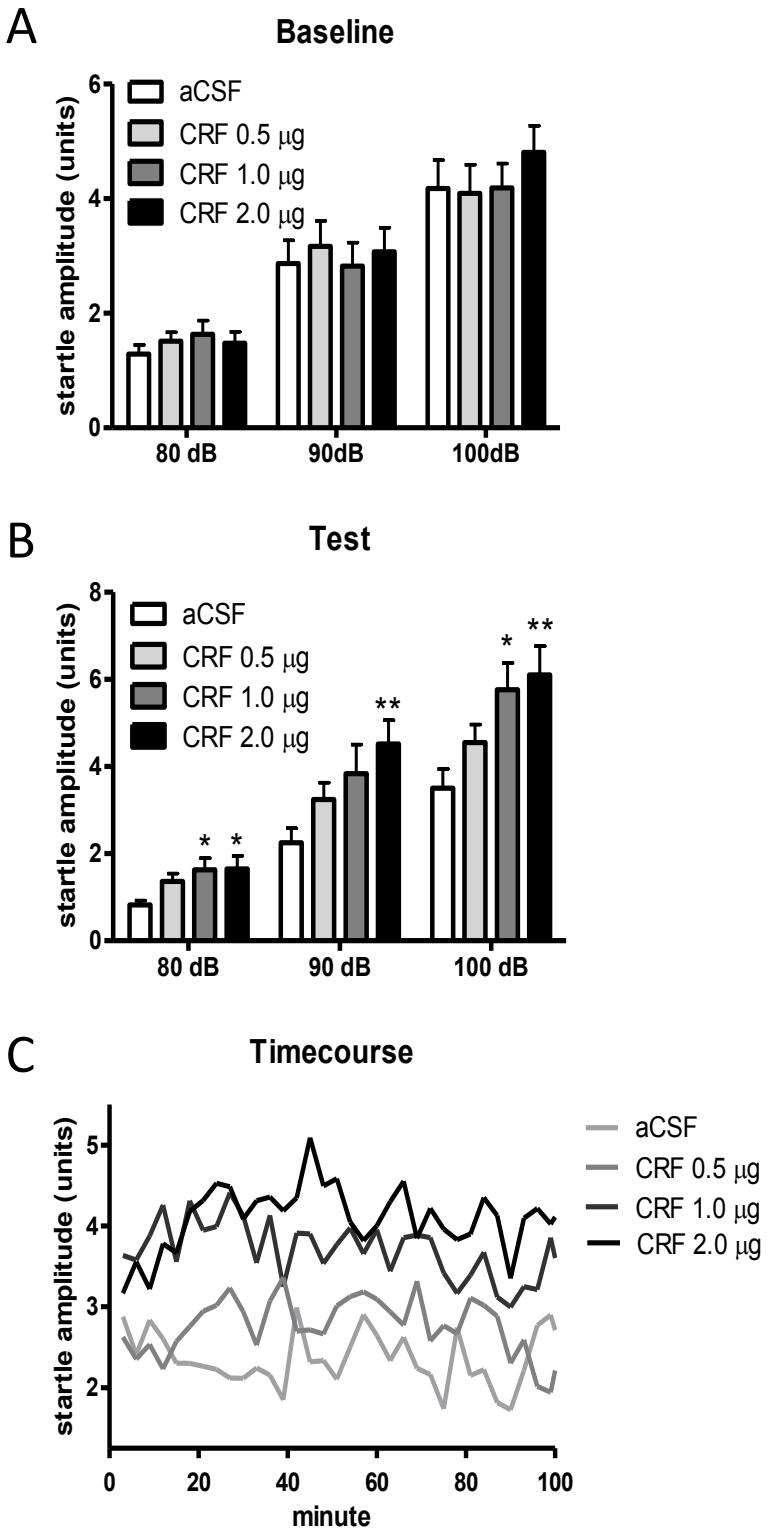


Figure 2.1 Representative cresyl-violet stained photomicrograph of an ICV cannula tract. Only mice with cannula tips within the lateral ventricle (LV) were included for analysis. ICV, intracerebroventricular

CRF produced dose-dependent increases in startle reactivity (**Fig. 2.2**). Prior to CRF infusion, mice were given a baseline startle test and were matched into groups with equivalent levels of startle ($F[3,29]=0.19$, NS) across three sound intensities (80, 90, 100 dB; as expected, there was a main effect of dB ($F[2,58]=147.23$, $P<0.001$; **Fig. 2.2A**). Infusions of CRF increased startle amplitude and this increase was dependent on an interaction of CRF dose (0.0, 0.5, 1.0 or 2.0 µg) and dB level (80, 90 and 100 dB; $F[6, 56]=3.35$, $P<0.01$). Main effects of dB ($F[2,58]=196.10$, $P<0.001$) and CRF ($F[3,29]=4.93$, $P<0.01$) were also observed. *Post hoc* analyses of between-subject effects at each dB level revealed that 2.0 µg CRF significantly increased startle at all dB levels, while 1.0 µg significantly enhanced startle at 80 and 100 dB (*P's*<0.05) (**Fig. 2.2B**). In contrast, 0.5 µg had no significant effects. Increases in startle were sustained throughout the 100 min test session (**Fig. 2.2C**).

Figure 2.2 Dose response of CRF on startle amplitude. Mice were infused with1.0 µL of vehicle (aCSF) or CRF (0.5, 1.0, or 2.0 µg) and startle amplitude data were collected across a 104 min test session. Startle bursts of 80, 90 or 100 dB were delivered every 30 sec throughout the session in a semi-random order. ***A***. Mice were matched into groups showing equivalent levels of baseline startle (n=8-9). ***B.*** CRF increased startle amplitude in a dose-dependent manner. ***C***. Time course of CRF's effect on startle amplitude across the entire test session. Data were analyzed by ANOVA followed by Bonferroni *post hoc* tests. **P*<0.05, ***P*<0.01 compared to aCSF control group. aCSF, artificial cerebrospinal fluid

Figure 2.2 (Continued) Dose response of CRF on startle amplitude



To study the effects of KOR antagonism on CRF-enhanced startle, mice were infused with 1.0 µg of CRF, the lowest dose that significantly increased startle. During surgery to implant the ICV guide cannula, mice were injected with the long-acting KOR antagonist JDTic (10 or 30 mg/kg, IP). Data for each dose of JDTic were analyzed separately. Mice were matched into groups with equivalent levels of baseline startle before surgery. No difference in baseline startle reactivity was observed between aCSF and CRF groups following 10 mg/kg JDTic pretreatment, but before CRF infusion ($F[1,36]=0.50$, NS; **Fig. 2.3A**). Startle amplitude increased as a function of dB level as demonstrated by a main effect of dB ($F[2,68]=227.99$, $P<0.001$). Further, JDTic at 10 mg/kg had no effect alone on baseline startle ($F[1,36]= 0.45$, NS). During the test session, mice received 1.0 µL infusions of CRF or vehicle and were immediately tested. Data from the test session revealed a CRF x dB interaction ($F[2, 68]=7.67$, $P<0.01$; **Fig. 2.3B**). Main effects of dB ($F[2,68]=197.73$, $P<0.001$) and CRF ($F[1,34]=19.73$, $P<0.001$) were also observed. There were no interactions of JDTic pretreatment (10 mg/kg) with any other factor, nor a main effect of JDTic. The effect of JDTic on KOR agonist-induced analgesia was tested the following day using the warm water tail-flick assay (**Fig. 2.3C,D**). Tail withdrawal latencies were collected at baseline prior to IP injection with U50,488 (15 mg/kg) and again 30 min following injection. Tail withdrawal latencies depended on both a JDTic x U50,488 ($F[1,34]=5.95$, $P<0.05$; **Fig. 2.3C**) and CRF x U50,488 ($F[1,34]=4.94$, $P<0.05$; **Fig. 2.3D**) interaction, whereas no 3-way interaction was observed. When data were collapsed across ICV treatment, *post hoc* within-group analysis demonstrated a significant increase in tail withdrawal latency in vehicle but not JDTic pretreated mice ($P<0.001$). No differences in baseline tail withdrawal latencies were detected in between-group analysis; however, there was a

significant difference between vehicle and JDTic pretreatment group latencies following U50,488 treatment (P<0.05). When data from the same studies were collapsed across JDTic treatment, analysis revealed no differences between aCSF or CRF treated mice either at baseline or following U50,488; however, while aCSF infused mice showed an expected increase in latency following U50,488 (P<0.01), this increase was not observed in the CRF group. Since the goal of our study was to prevent CRF-induced effects with JDTic pretreatment, we further analyzed the tail-flick data excluding all aCSF mice to determine whether a lack of JDTic effect in CRF-enhanced startle may be due to insufficient blockade of KORs. In this analysis, there was a main effect of U50,488 treatment (F[1,18]=4.88, P<0.05; data not shown), but no interaction with JDTic as would be expected if KOR function was antagonized. We therefore repeated the study using a higher dose of JDTic (30 mg/kg).

Tests at the 30 mg/kg dose of JDTic similarly revealed no effect of JDTic on baseline startle reactivity (F[1,37]=0.09, NS; **Fig. 2.4A**). Further, there were no differences in startle magnitude between aCSF and CRF groups before infusion (F[1,37]=0.01, NS; **Fig. 2.4A**). As expected, a main effect of dB (F[2,74])=235.37, P<0.001) indicated startle amplitude was dependent on dB level at baseline. In the test session, JDTic attenuated CRF-induced increases in startle. This effect was dependent on dB level, as indicated by a JDTic x CRF x dB interaction (F[2,74]=3.93, P<0.05; **Fig. 2.4B**). Main effects of dB (F[2,74]=262.32, P<0.001) and CRF (F[1,37]=22.71, P<0.01) also emerged as well as a JDTic x CRF interaction (F[1,37]=4.97, P<0.05). Follow-up two-way ANOVAs at each dB level revealed that CRF effects were dependent on JDTic pretreatment at 90 dB (F[1,37]=4.25, P<0.05) and 100 dB (F[1,37]=4.79, P<0.05). At 80

dB, CRF increased startle amplitude (F[1,37]=51.25, *P*<0.001; however, JDTic failed to reduce this response. *Post hoc* analyses demonstrated that vehicle pretreated mice infused with CRF had significantly higher startle reactivity at 90 and 100 dB compared to vehicle and JDTic pretreated mice infused with aCSF (*P*<0.01) and more importantly, to JDTic pretreated mice infused with CRF (*P*<0.05). Following the test session, KOR agonist-induced analgesia was tested in the tail-flick as above (**Fig. 2.4C**). JDTic pretreatment reduced U50,488-induced increases in tail withdrawal latency as reflected by a JDTic x U50,488 interaction (F[1,37]=11.39, *P*<0.01). Vehicle pretreated mice had a significant increase in tail withdrawal latency following U50,488 compared to baseline (within-subjects *post hoc* analysis, *P*<0.001). This effect was not seen in JDTic pretreated mice (*P*=0.66). Further, U50,488 significantly increased tail withdrawal latencies in vehicle compared to JDTic pretreated mice (*P*<0.001). JDTic on its own did not have an effect on baseline tail withdrawal latency (*P*=0.55). These same effects were still observed when aCSF infused mice were removed from the analysis (F[1,21]=5.38, *P*<0.05). Tail withdrawal latencies were significantly increased in vehicle pretreated (*P*<0.001) but not JDTic pretreated mice (*P*=0.67) following U50,488 compared to baseline. Further, tail withdrawal latencies were significantly higher following U50,488 injection in vehicle compared to JDTic pretreated mice (*P*<0.01).

Figure 2.3 Effect of JDTic (10/mg/kg) on CRF-enhanced startle. Mice were implanted with ICV cannulae, administered JDTic (10 mg/kg), and allowed to recover. ***A.*** Groups were matched for equivalent baseline startle. (n=9-10). On the test day mice received CRF (1µg) or vehicle and were immediately placed in the startle apparatus. ***B.*** JDTic did not significantly alter CRF-induced increases in startle amplitude at any decibel level. ***C-D.*** Effect of JDTic pretreatment on latency to withdraw the tail (in sec) in the tail-flick assay at baseline or 30 min after administration of the KOR agonist U50,488 (15 mg/kg, IP). ***C.*** U50,488 increased tail withdrawal latencies in vehicle, but not JDTic pretreated mice. ***D.*** Mice infused with aCSF showed an increase in tail withdrawal latencies following U50,488, which is absent in CRF infused mice. Data were analyzed by ANOVA followed by Bonferroni *post hoc* test as appropriate. **P*<0.05 between group comparison, ****P*<0.001 within group comparison. Veh, vehicle

Figure 2.3 (Continued) Effect of JDTic (10/mg/kg) on CRF-enhanced startle

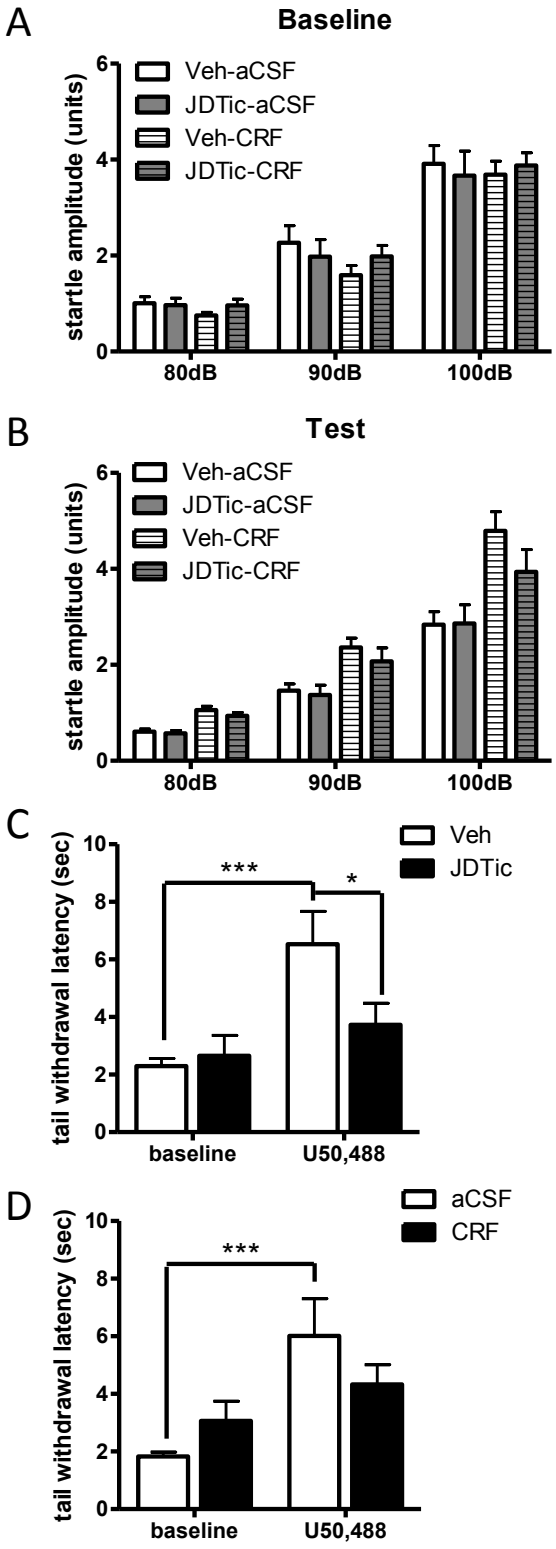
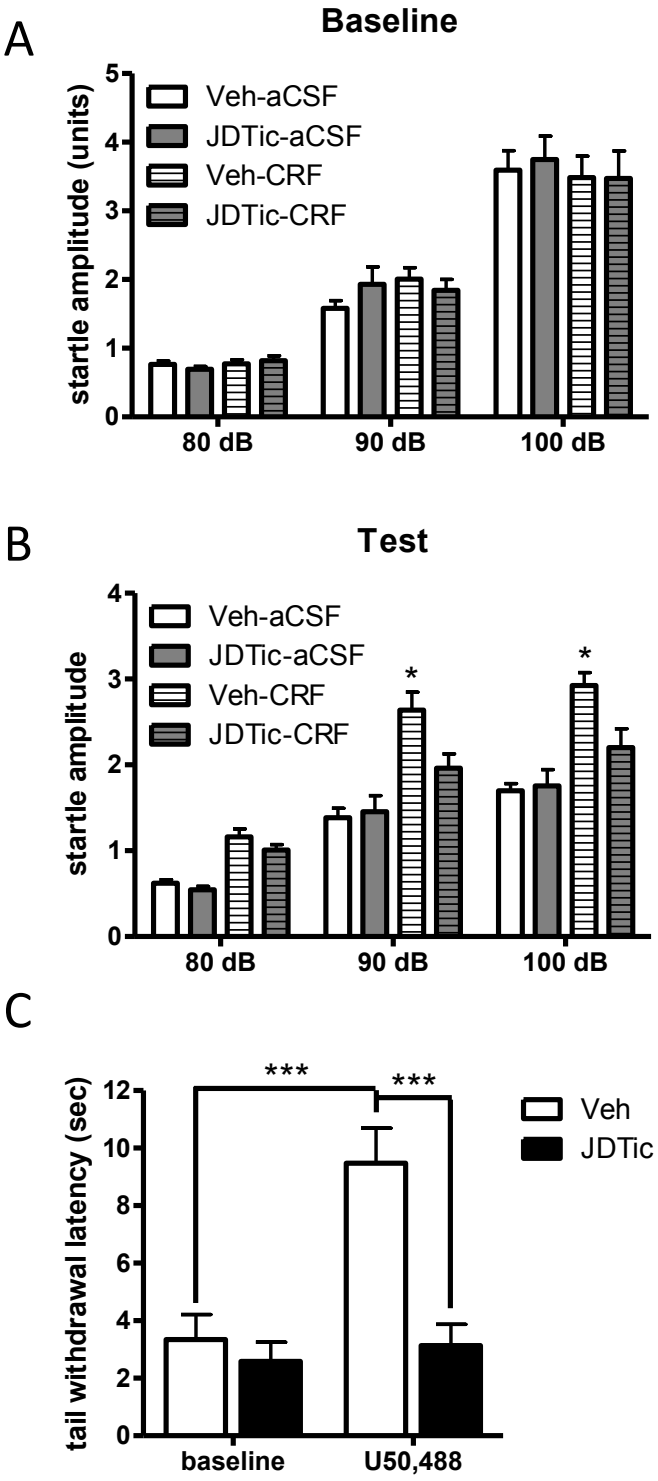


Figure 2.4 Effect of JDTic (30/mg/kg) on CRF-enhanced startle. ***A.*** Groups were matched for equivalent baseline startle. (n=9-12). On the test day mice received CRF (1µg) or vehicle and were immediately placed in the startle apparatus. ***B.*** JDTic significantly reduced CRF-enhanced startle at 90 and 100 dB. ***C.*** Effect of JDTic pretreatment on latency to withdraw the tail (in sec) in the tail-flick assay at baseline or 30 min after administration of the KOR agonist U50,488 (15 mg/kg, IP). U50,488 increased tail withdrawal latencies in vehicle, but not JDTic pretreated mice. Data were analyzed by ANOVA followed by Bonferroni *post hoc* test as appropriate. **P*<0.05, ****P*<0.001

Figure 2.4 (Continued) Effect of JDTic (30/mg/kg) on CRF-enhanced startle



To investigate the effects of JDTic on CRF-induced changes in brain activation, c-Fos immunohistochemistry was performed as a marker of neuronal activation. Mice were pretreated with JDTic (30 mg/kg, IP) during ICV surgery and perfused 8 days later following aCSF or CRF (1.0 µg) infusion. Brain regions analyzed are shown in **Fig. 2.5**. and their abbreviations are listed in **Table 2.1**. In the VTA, CRF effects depended upon an interaction with JDTic ($F[1,33]=4.94$, $P<0.05$). *Post hoc* analysis revealed that JDTic-treated mice had significantly fewer CRF-induced c-Fos immunoreactive cells compared to vehicle treatment ($P<0.05$, **Fig. 2.6**). Example photomicrographs show reduced c-Fos cell labeling in the JDTic /CRF group compared to the vehicle/CRF group (**Fig. 2.6A**). In the dDG, a main effect of JDTic was observed ($F[1,33]=6.48$, $P<0.05$), as JDTic reduced c-Fos cell numbers in both aCSF- and CRF-treated mice (**Fig. 2.7**). **Table 2.1** includes the mean number of c-Fos positive cells for all regions analyzed. *P*-values listed are for the JDTic x CRF interaction. A significant main effect of CRF treatment was observed in all regions including the AcbC ($F[1,33]=25.82$, $P<0.001$), AcbSh ($F[1,33]=230.44$, $P<0.001$), BLA ($F[1,33]=56.81$, $P<0.001$), CeA ($F[1,33]=1290.62$, $P<0.001$), BNST ($F[1,33]=1470.55$, $P<0.001$), IL ($F[1,33]=109.04$, $P<0.001$), Pir ($F[1,33]=36,61$, $P<0.001$), DRD ($F[1,33]=138.03$, $P<0.001$), dCA1 ($F[1,33]=83.43$, $P<0.001$), dCA2 ($F[1,33]=164.55$, $P<0.001$), dCA3 ($F[1,33]=288.09$, $P<0.001$), dDG ($F[1,33]=67.29$, $P<0.001$), dStr ($F[1,33]=12.90$, $P<0.001$), and VTA ($F[1,33]=58.51$, $P<0.001$).

Figure 2.5 Location of regions of interest analyzed for c-Fos immunoreactivity. Areas are demarcated by boxes on representative atlas images of the mouse brain (figures 17, 22, 30, 45, 46, 57 and 69 in Paxinos and Franklin (2001)). Anteroposterior distance from Bregma is indicated below each image. The definitions of abbreviations used are located in **Table 2.1**.

Figure 2.5 (Continued) Location of regions of interest analyzed for c-Fos immunoreactivity

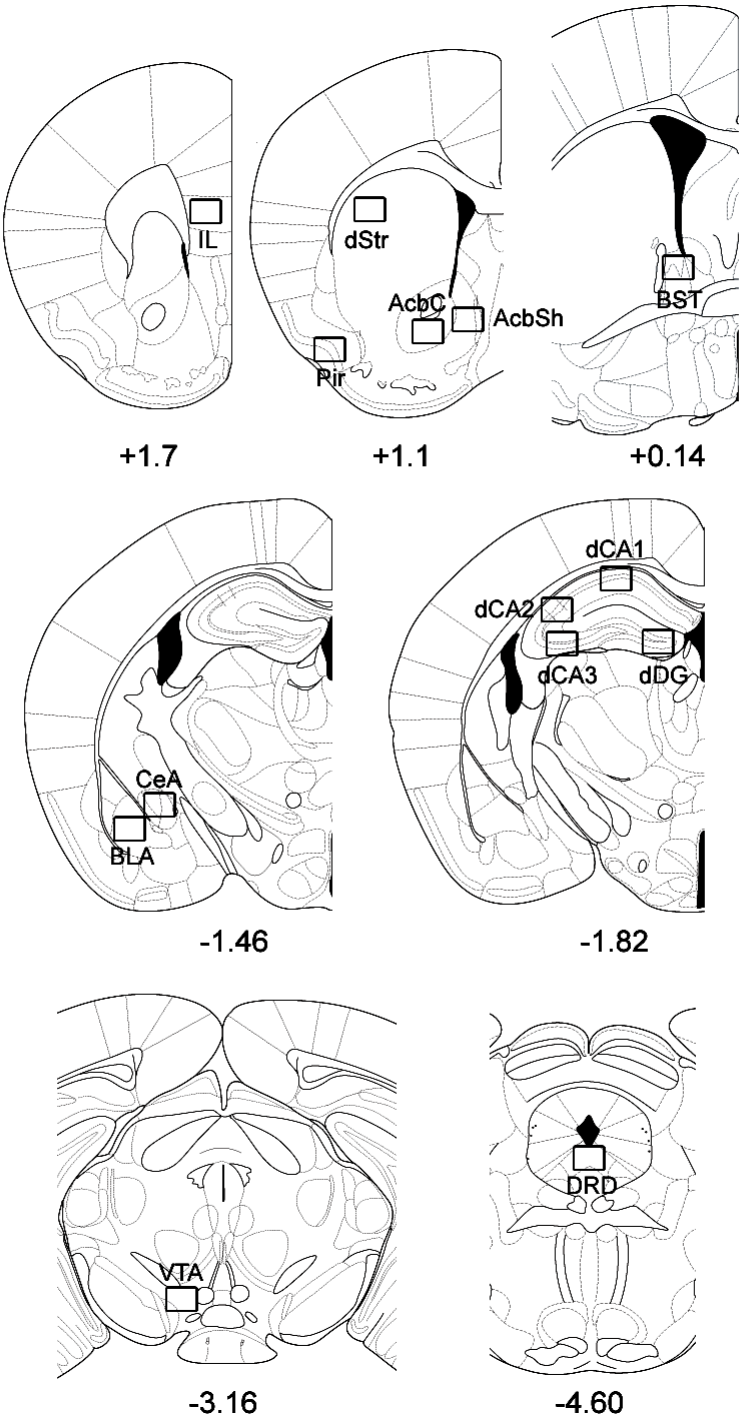


Figure 2.6 Effect of JDTic on c-Fos immunoreactivity in the VTA. ***A.*** Representative photomicrographs of c-Fos staining in the VTA at 20x. Left panels represent vehicle pretreated mice while right panels indicate JDTic (30 mg/kg, IP) pretreatment. Top panels are from aCSF infused mice while images in bottom panels are from CRF treated subjects. Dorsal is at the top and medial is to the left. Bar indicates 200 μm. Dashed box is representative of area counted at 40x for analysis. ***B.*** JDTic significantly decreased CRF-induced increases in c-Fos counts in the VTA. Data were analyzed by ANOVA followed by Bonferroni *post hoc* test. **P*<0.05, ***P*<0.01. VTA, ventral tegmental area

Figure 2.6 (Continued) Effect of JDTic on c-Fos immunoreactivity in the VTA

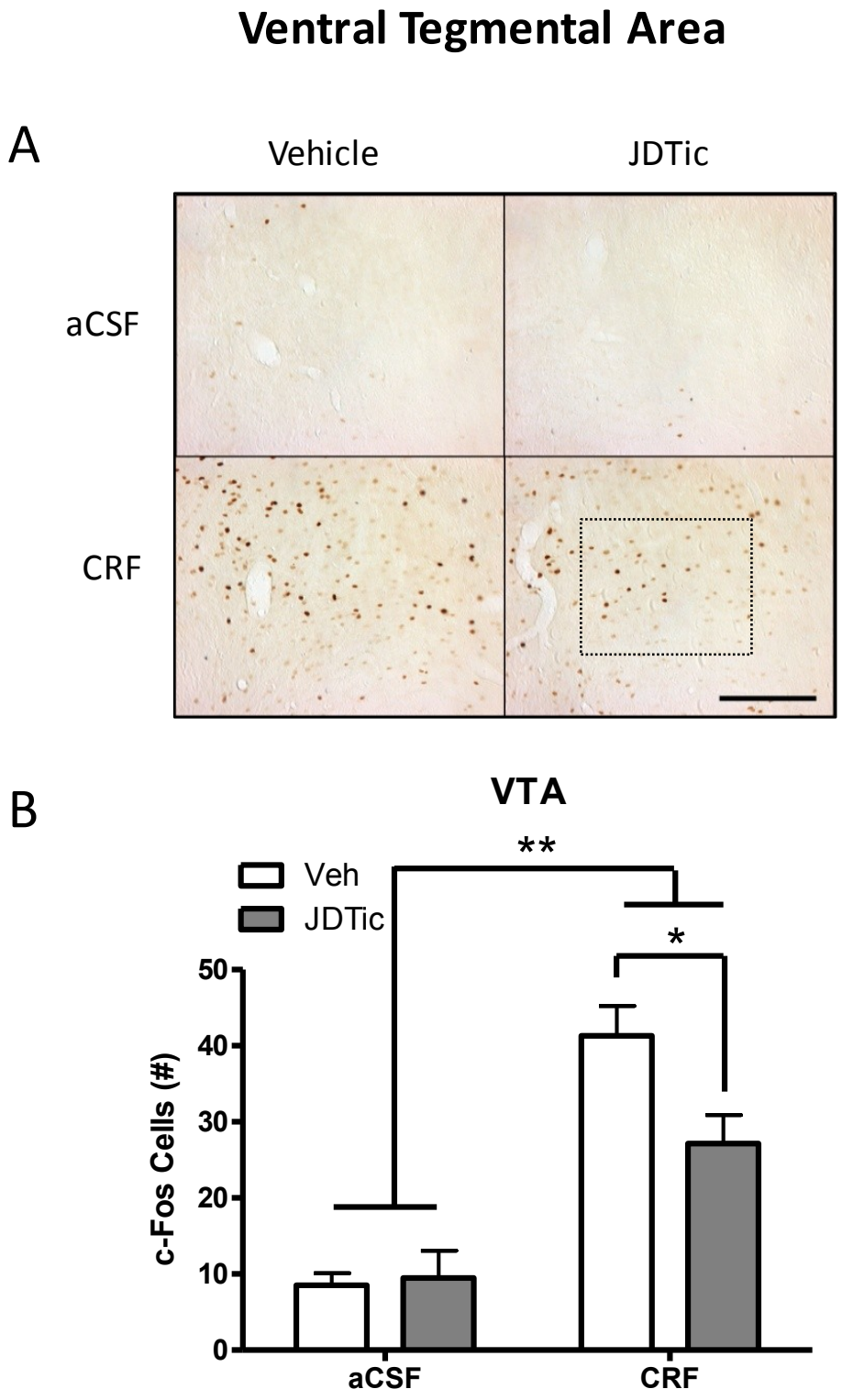


Figure 2.7 Effect of JDTic on c-Fos immunoreactivity in the dDG. ***A.*** Representative photomicrographs of c-Fos staining in the dDG at 20x. Left panels represent vehicle pretreated mice while right panels indicate JDTic pretreatment. Top panels are from aCSF infused mice while images in bottom panels are from CRF treated subjects. Dorsal is at the top and medial is to the left. Bar indicates 200 μm. Dashed box is representative of area counted at 40x for analysis. ***B.*** JDTic significantly decreased c-Fos counts in the dDG regardless of CRF infusion. Data were analyzed by ANOVA followed by Bonferroni *post hoc* test. **P*<0.05, ****P*<0.001. dDG, dorsal dentate gyrus of the HIP

Figure 2.7 (Continued) Effect of JDTic on c-Fos immunoreactivity in the dDG

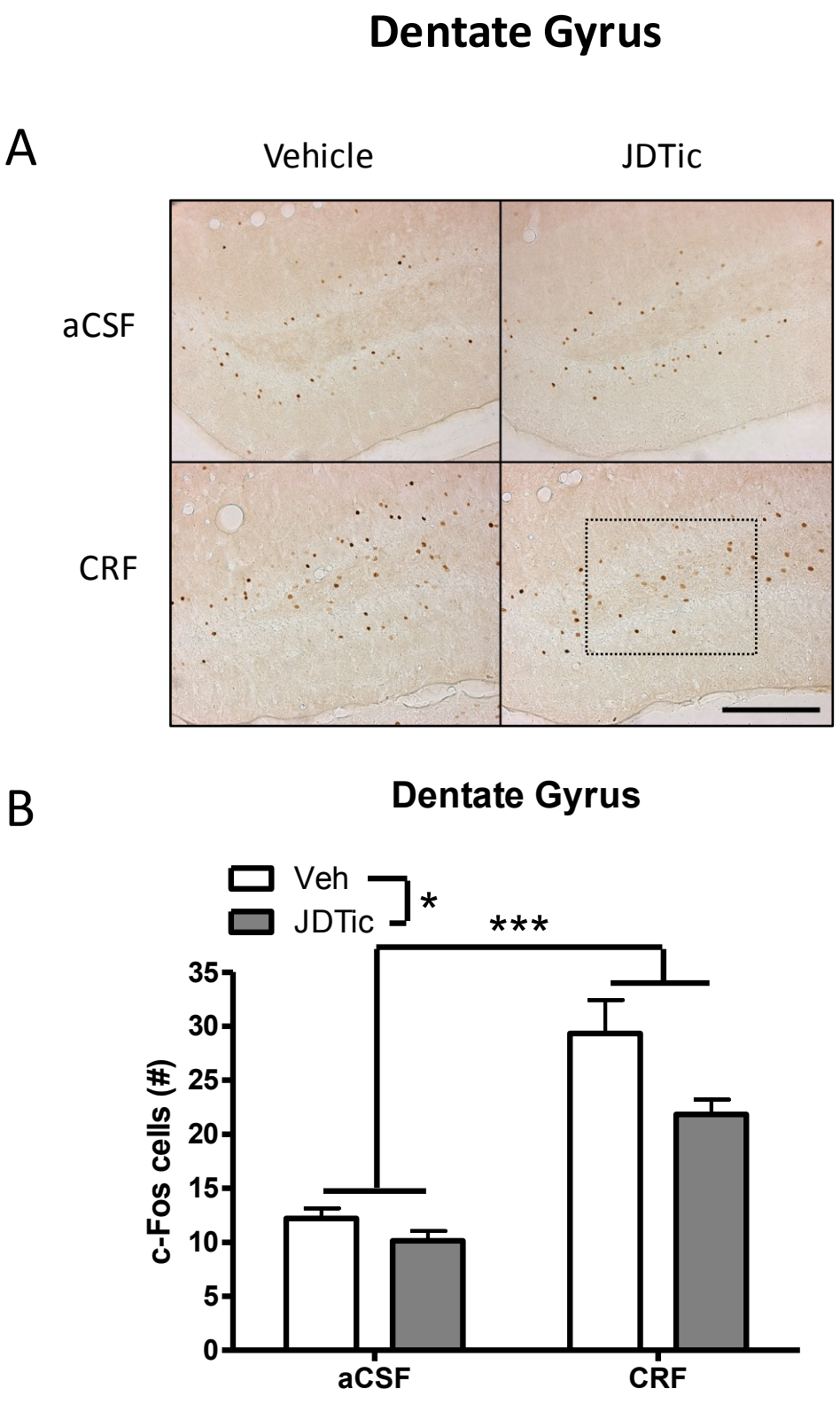


Table 2.1 Effects of JDTic on C-Fos expression after infusion of CRF (1.0 µg, ICV)

Brain Area	Sub-division	Abbr.	c-Fos Cell # (mean ± SEM)				*P*-value*
		IP **ICV**	Saline aCSF	JDTic aCSF	Saline CRF	JDTic CRF	
Accumbens nuclei	Core	AcbC	0.6 ± 0.2	1.5 ± 0.4	13.4 ± 3.2	17.8 ± 4.4	0.549[a]
	Shell	AcbSh	6.2 ± 1.3	9.9 ± 1.9	52.2 ± 4.1	54.3 ± 3.5	0.793[a]
Amygdaloid nuclei	Basolateral	BLA	5.0 ± 1.8	4.3 ± 1.0	19.5 ± 2.0	18.1 ± 2.3	0.865[a]
	Central	CeA	4.5 ± 1.3	6.8 ± 1.6	161.0 ± 6.1	164.6 ± 6.0	0.887[a]
Bed nucleus of the stria terminalis	Lateral Division	BNST	10.8 ± 1.8	14.3 ± 2.7	132.7 ± 3.9	135.5 ± 3.7	0.897[a]
Cortex	Infralimbic	IL	24.1 ± 5.4	28.8 ± 4.9	79.2 ± 6.3	83.7 ± 4.3	0.990[a]
	Piriform	Pir	16.8 ± 6.3	25.2 ± 4.1	97.6 ± 16.8	78.4 ± 12.0	0.221[a]
Dorsal raphe nucleus	Dorsal part	DRD	16.4 ± 2.3	19.3 ± 2.4	50.3 ± 4.0	55.2 ± 2.9	0.727[a]
Dorsal hippocampus	field CA1	dCA1	2.9 ± 1.4	2.3 ± 0.6	43.8 ± 5.9	34.3 ± 5.0	0.279[a]
	field CA2	dCA2	3.2 ± 1.3	4.9 ± 1.7	34.0 ± 3.0	30.0 ± 2.3	0.204[a]
	field CA3	dCA3	5.8 ± 1.2	5.9 ± 1.0	27.6 ± 0.9	26.1 ± 1.6	0.533[a]
	dentate gyrus	dDG	12.2 ± 0.9	10.1 ± 0.9	28.4 ± 2.9	21.8 ± 1.4	0.194[a,b]
Striatum	Dorsal	dStr	0.2 ± 0.1	0.3 ± 0.1	8.3 ± 2.8	8.3 ± 3.3	0.978[a]
Ventral tegmental area		VTA	8.5 ± 1.6	9.5 ± 3.6	40.7 ± 3.5	27.2 ± 3.7	0.033[a]

*p-value for JDTic x CRF interaction

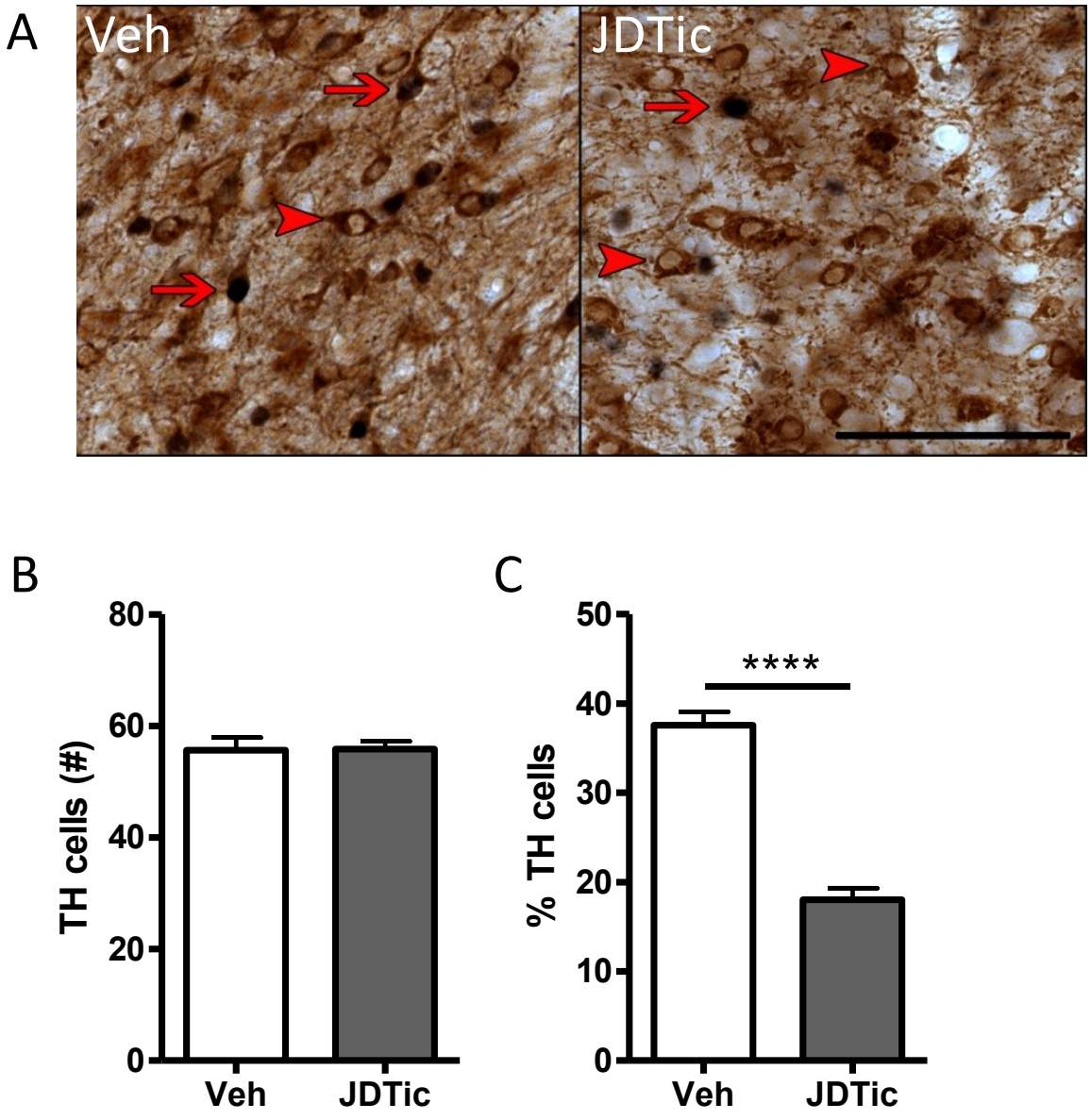
[a] Significant main effect of CRF, $p \leq 0.001$, [b] Significant main effect of JDTic, $P=0.016$

Mice were pretreated with vehicle or JDTic (30 mg/kg, IP) before infusion of aCSF or CRF (1.0 µg) n=9-10 per group

To identify the cell types affected by JDTic pretreatment, double-labeling experiments for c-Fos and TH were carried out in alternate sections of the VTA. Since the decrease in c-Fos cells was only apparent in CRF-treated mice, sections from aCSF-treated mice were not processed for immunohistochemistry. The VTA predominantly consists of dopaminergic cells, so an antibody to TH, which labels this neuronal population in the VTA, was used to determine if c-Fos reductions were occurring in DA neurons. JDTic significantly reduced the number of c-Fos positive cells that colocalized with TH (**Fig. 2.8**).

Figure 2.8 Effect of JDTic on c-Fos immunoreactivity in DA cells of the VTA. ***A.*** Representative photomicrographs of c-Fos and TH labeling in CRF-infused mice treated with saline or JDTic (30 mg/kg). Arrows indicate c-Fos and TH double-labeled cells while arrowheads point to TH alone. ***B.*** The number of TH-positive cells counted for saline and JDTic groups did not differ. ***C.*** The percent of TH cells colabeled with c-Fos was significantly reduced in mice pretreated with JDTic compared to controls. Data were analyzed by Student's t-test. ****P<0.0001 Scale bar indicates 100 μm. DA, dopamine

Figure 2.8 (Continued) Effect of JDTic on c-Fos immunoreactivity in DA cells of the VTA



Discussion

Central infusions of CRF dose-dependently increased acoustic startle reactivity; doses of 1.0 and 2.0 µg CRF significantly increased startle amplitude compared to vehicle. Increases in acoustic startle reactivity following 1.0 µg infusions of CRF were nominally attenuated by a single pretreatment with the long-lasting KOR antagonist JDTic at 10 mg/kg and significantly blocked at 30 mg/kg, IP. This same dose of CRF induced neuronal activation throughout the brain as measured by c-Fos immunoreactivity. Pretreatment with JDTic (30 mg/kg) blocked CRF-induced increases in c-Fos cell counts in the VTA while causing a more general reduction in c-Fos positive cell counts, regardless of CRF treatment, in the dDG of the HIP. These results suggest that KOR activation mediates CRF effects on startle and implicates the VTA and dDG in these effects.

The general goal of these experiments was to examine the effects of KOR antagonism on CRF-enhanced startle and brain c-Fos activation in mice. After characterizing the CRF dose-effect function, we administered CRF at 1.0 µg (ICV) for subsequent studies, since this was the lowest of the doses tested to elicit significant increases in startle amplitude. Use of this intermediate dose enabled us to detect both decreases and increases in CRF-enhanced startle following JDTic pretreatment. It also enables us to compare our results with those of other reports testing CRF-induced behavioral effects in mice including elevated startle (Swiergiel and Dunn, 1999; Risbrough et al., 2004; Risbrough and Geyer, 2005; Land et al., 2008; Bruchas et al., 2009), suggesting this is an appropriate dose to investigate compounds with anxiolytic-like effects, although

doses lower than 1.0 µg ICV can induce changes in other behavioral tests (see Dunn and Berridge, 1990).

Prior to CRF infusion, JDTic pretreatment at either 10 or 30 mg/kg did not have an effect on baseline startle responding in mice. This finding is consistent with previous reports indicating lack of effects of JDTic (Knoll et al., 2007) or norBNI (Bortolato et al., 2005; Tejeda et al., 2010a)—another highly selective KOR antagonist with similar pharmacodynamics to JDTic (Munro et al., 2012)—on baseline startle. Since JDTic alone produced no appreciable effects on startle, we sought to determine if KOR antagonism would specifically alter CRF responses. In our initial study, JDTic at 10 mg/kg did not significantly reduce increases in startle amplitude in response to CRF. This likely indicates the use of an insufficient dose of JDTic; indeed, this dose was selected on the basis of studies in rats (Knoll et al., 2007). Because JDTic is a highly selective and long-lasting KOR antagonist with a slow onset of action (Carroll et al., 2004; Beardsley et al., 2005), we administered it at the time of surgery, 7 days prior to testing. This also enabled drug administration during anesthesia, thereby avoiding the stress of an IP injection in conscious mice. Single injections of JDTic have been shown to have a duration of action in mice of 14-21 days in a warm water tail-flick assay similar to that used in the present work (Bruchas et al., 2007b; Melief et al., 2011), and we have reported a similar extended time course in rats (Van't Veer et al., 2012). Here we report that 10 mg/kg JDTic blocks U50,488 effects 8 days following administration; however, since the comparison of most interest to us was between the vehicle and JDTic groups that received CRF, an analysis of the tail-flick data comparing only these groups was performed. This revealed an effect of U50,488 treatment, but no interaction

with JDTic, suggesting that KOR antagonism was insufficient at this time point and may explain why JDTic did not significantly attenuate CRF effects. As such, we conducted another study of CRF-enhanced startle following pretreatment with 30 mg/kg JDTic. Dose effects of 10 and 30 mg/kg JDTic have been reported in studies of footshock-induced reinstatement of cocaine administration in rats (Beardsley et al., 2005), suggesting that higher doses might be tolerable in mice. Indeed, 30 mg/kg JDTic significantly decreased startle amplitude in CRF treated mice compared to vehicle controls at 90 and 100 dB without having any effect on baseline startle. Further, U50,488-induced antinociceptive effects were significantly reduced in JDTic pretreated mice, even when excluding the aCSF treated mice. These data confirm a role for KORs in the anxiogenic-like effects of CRF on startle. It is important to note that the 10 mg/kg and 30 mg/kg studies were conducted as entirely separate studies. We designed the studies in this way because we have noted cohort and even seasonal differences in baseline and CRF-enhanced startle reactivity. Indeed, there were some cohort differences in CRF-enhanced startle, highlighting the importance of including all of the respective control conditions in this second (JDTic, 30 mg/kg) experiment.

The mechanisms by which CRF and KOR systems interact are not fully understood. KORs and dynorphin are expressed in brain regions involved in anxiety (Fallon and Leslie, 1986; Mansour et al., 1995) which overlap with CRF and CRF-R expression (Merchenthaler, 1984; De Souza et al., 1985; Millan et al., 1986; Van Pett et al., 2000; Alon et al., 2009). Stress can elevate dynorphin expression and release (Przewlocki et al., 1987; Nabeshima et al., 1992; Shirayama et al., 2004; Chartoff et al., 2009), and central CRF can also stimulate dynorphin release (Song and Takemori, 1992) and

activate KOR receptors (Land et al., 2008; Bruchas et al., 2009). These findings suggest that at least some CRF effects are mediated by downstream KOR activation. To identify circuits in which relevant CRF and KOR interactions may occur, we used c-Fos immunolabeling as a marker of neuronal activation following CRF infusion in JDTic and vehicle pretreated mice. Our analysis focused on regions implicated in fear and anxiety-like behavior in animals and humans, and thus was extensive but not exhaustive. Infusions of CRF increased c-Fos cell counts in all regions analyzed, in agreement with other reports of activation within these same areas in rats (Andreae and Herbert, 1993; Imaki et al., 1993; Marrosu et al., 1996; Bittencourt and Sawchenko, 2000). In contrast, only c-Fos counts within the VTA and dDG were affected by JDTic pretreatment.

In the VTA, JDTic specifically reduced increases in c-Fos cell counts following CRF infusion, while having no effect on counts in the control (aCSF) condition. The majority of this reduction occurred in TH+ (dopaminergic) neurons, suggesting that the ability of JDTic to decrease CRF-enhanced startle may rely on reductions of CRF-induced modulation of DA activity (Ungless et al., 2003; Wanat et al., 2008). Indeed, electrical stimulation of the VTA increases startle amplitude while axon-sparing lesions of this area reduce fear-potentiated startle (Borowski and Kokkinidis, 1996). Further, intra-VTA infusions of the DA autoreceptor agonist quinpirole, which inhibits DA release, attenuate conditioned fear (Borowski and Kokkinidis, 1996; Munro and Kokkinidis, 1997; Nader and LeDoux, 1999; Gifkins et al., 2002). Collectively, these data suggest that reductions in CRF-induced VTA activity and subsequent DA release may have anxiolytic-like effects including attenuation of CRF-enhanced startle, and provide a

rationale for future work that focuses on the role of KORs expressed on VTA DA cells in anxiety-related behavior.

In the dDG, JDTic reduced c-Fos labeling regardless of whether mice received ICV infusions of CRF or aCSF. These decreases may represent JDTic-mediated reductions in activation within circuits underlying CRF effects on startle. The startle reflex pathway is a simple circuit involving sound input reaching cochlear root neurons via the ear, subsequent activation of the caudal nucleus of the pontine reticular formation (PnC), and signal output via spinal motoneurons terminating at the neuromuscular junction and producing a fast muscle contraction (flinch) (Davis et al., 1982; Yeomans and Frankland, 1995). This response is thought to be modulated at the level of the PnC by cortical and limbic inputs. While the HIP had no direct projections to the PnC, lesions of the HIP can alter the acoustic startle reflex (Coover and Levine, 1972; Leaton, 1981; Walsh et al., 1986; Mickley and Ferguson, 1989; Caine et al., 1992). These data suggest that while the HIP is not directly involved in the startle reflex, it regulates startle-relevant circuits. Indeed, mutant mice with increased startle reactivity show increased HIP excitability (Fisahn et al., 2011). Higher HIP excitability has also been shown to correlate with stress-induced anxiety (Mucha et al., 2011). The present findings suggest that JDTic-induced decreases in putative neuronal activation may reduce HIP excitability in response to CRF and thus prevent increases in anxiety-like behavior, measured here as elevated startle. Although beyond the scope of the current work, electrophysiological studies may provide deeper insight on the mechanisms by which KOR antagonism affects this circuitry. In addition, other behavioral tests—specifically,

those that depend more directly on HIP function—may reveal a role for HIP KORs in anxiety-related behavior.

The present work confirms a role for KORs in stress-induced behavior by demonstrating that CRF-induced elevations of the acoustic startle reflex are attenuated by KOR antagonism. One of the hallmark signs of PTSD is an exaggerated startle reflex, which is often persistent and debilitating. In individuals suffering from PTSD, the CRF system is overactive, which is thought to be a primary contributing factor in this disorder (Arborelius et al., 1999; Kasckow et al., 2001). Our results suggest that KOR antagonists may be clinically useful to alleviate stress-induced behaviors including exaggerated startle and lead to greater improvements in patient treatment. The next chapter describes the role of KORs in another paradigm of stress-induced behavior—footshock potentiated startle—that similarly uses potentiation of the acoustic startle reflex as an index of anxiety-like behavior in mice, in order to extend our findings to a more naturalistic stress response. This paradigm also relies on context conditioning, a HIP-dependent task (Phillips and LeDoux, 1992) that will enable further characterization of KORs in this region.

Chapter 3

Antagonism of kappa-opioid receptors reduces footshock-potentiated startle

Ashlee Van't Veer[1], F. Ivy Carroll[2], William A. Carlezon, Jr.[1]

[1]Department of Psychiatry, Harvard Medical School, McLean Hospital, Belmont, MA 02478; [2]Research Triangle Institute, Research Triangle Park, NC

Author contributions: AVV and WAC designed the experiments. AVV conducted the experiments and analyzed the data. FIC synthesized and provided JDTic.

Abstract

Stress is a precipitating factor for depressive and anxiety disorders. In severe cases, exposure to contextual cues associated with traumatic stress is sufficient to trigger signs and symptoms of these disorders. Prior work has shown that some effects of stress including dysphoria, anhedonia, and anxiogenic-like behaviors are mediated by the KOR system. Blockade of KOR signaling with KOR antagonists produces antidepressant-like effects and anxiolytic-like effects in conditioned and unconditioned fear models, and reduces the effects of stress and the stress-related neuropeptide CRF. The present studies were designed to extend these findings using footshock-potentiated startle, a model of contextual conditioning. Mice were habituated to the startle apparatus and matched into groups with equivalent levels of baseline startle before drug treatment and testing. They were then pretreated with the prototypical, long-lasting KOR antagonist JDTic (10 or 30 mg/kg) and tested 24 hr later. KOR antagonism significantly decreased footshock effects on startle. To identify brain regions that may contribute to this effect, c-Fos immunohistochemistry was performed to map neuronal activation patterns. In control mice, footshock induced a small but significant increase in c-Fos cell counts in the highly stress-responsive PVN. However, analysis in the HIP and VTA did not reveal changes in neuronal activation following footshock or differences in JDTic-pretreated mice compared to controls. Thus we could not establish any associations between behavioral endpoints and neural activation in the brain regions studied. Regardless, this work confirms a role for KORs in stress-induced increases in startle.

Introduction

Severe or sustained stress often coincides with the development of anxiety disorders and clinical depression (Kessler, 1997; Kendler et al., 1999; Pine et al., 2002). In laboratory settings, procedures involving stress exposure often trigger depressive- and anxiety-like behaviors in rodents (Platt and Stone, 1982; Zacharko and Anisman, 1991; Keeney and Hogg, 1999; Aisa et al., 2008; Slattery et al., 2012). Stress-like effects can also be precipitated by central CRF infusion (Britton et al., 1982; Dunn and File, 1987; Stenzel-Poore et al., 1994; van Gaalen et al., 2002; Swiergiel et al., 2008). CRF is a key regulator of the stress response and dysfunction of CRF systems is thought to underlie human depressive and anxiety disorders (Nemeroff et al., 1984; Widerlov et al., 1988; Arborelius et al., 1999; Kasckow et al., 2001). One clinical sign of these disorders is exaggerated startle reactivity, particularly in response to environmental (contextual) cues previously associated with stress (Grillon and Baas, 2003; Ray et al., 2009). In rodents, potentiation of the acoustic startle response can be achieved by central infusions of CRF (Swerdlow et al., 1986; Liang et al., 1992), although a direct link between increased startle and CRF levels has not been established in humans. Nevertheless, determining the downstream mediators of stress/CRF effects on startle may provide important targets for the development of drugs to alleviate or prevent the deleterious effects of stress.

It has been demonstrated that some of the key effects of stress and CRF are mediated by the KOR system, which comprises KORs and their endogenous ligand dynorphin (Chavkin et al., 1982). Stress induces the release of dynorphin (Nabeshima et al., 1992), which activates KORs and produces aversive behaviors (Land et al., 2008).

KOR agonists can mimic the effects of stress (McLaughlin et al., 2006a), suggesting KOR activation is a critical step in producing the behavioral consequences of stress. Conversely, KOR antagonists are effective at blocking the effects of stress such as stress-induced analgesic behavior (Takahashi et al., 1990; McLaughlin et al., 2003a; McLaughlin et al., 2006b), learned helplessness (Pliakas et al., 2001; Newton et al., 2002; Mague et al., 2003), and drug reinstatement (Beardsley et al., 2005; Carey et al., 2007). KOR antagonists also produce anxiolytic-like effects in models of learned and unlearned fear (Knoll et al., 2007), and can attenuate the anxiogenic-like and dysphoric effects of CRF (Chapter 2; Land et al., 2008; Bruchas et al., 2009; Van't Veer et al., 2012). Thus disruption of KOR function may diminish the aversive or maladaptive consequences of stress exposure.

Fear-like states in rodents can be achieved through brief presentation of mild footshock. The context—which may comprise visual, tactile, auditory, and olfactory cues—in which footshock occurs becomes associated with this aversive event, and subsequent re-exposure to the cues alone can potentiate the startle reflex (Boulis and Davis, 1989; Davis, 1989; Hitchcock et al., 1989; McNish et al., 1997; Richardson, 2000; Sasaki and Hanamoto, 2007; Risbrough et al., 2009). Increased startle reactivity following shock is also observed in humans (Greenwald et al., 1998), suggesting evolutionarily-conserved mechanisms. Footshock has been shown to induce CRF release (Wang et al., 2005) while disrupting CRF receptor activation can block the behavioral effects of footshock (Ho et al., 2001; Bakshi et al., 2002; Le et al., 2002). Further, footshock-potentiated startle is abolished in CRF receptor knockout mice (Risbrough et al., 2009) suggesting CRF signaling and consequent downstream events are necessary for the anxiogenic-

like effects of footshock. Contextual conditioning is thought to depend upon the HIP (Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Rudy et al., 2002), a brain area in which the KOR antagonist JDTic affects neural activity, as reflected by c-Fos activation (Chapter 2). As such, based on our previous work, we hypothesized that the ability of footshock to potentiate the acoustic startle response would be dependent on KOR activation in the HIP.

The present studies investigated the effects of KOR antagonism on anxiety-like behavior, measured as an increase in the acoustic startle response following presentation of footshock (Davis, 1989). Mice were given a single injection of the long-acting KOR antagonist JDTic (Carroll et al., 2004) and tested for footshock-potentiated startle 24 hr later. The following day they were again subjected to startle testing in the context where footshock occurred. In these same mice, KOR antagonism was directly examined in the warm water tail-flick assay by the ability of JDTic to block the analgesic effects of a KOR agonist (McLaughlin et al., 2003a; Van't Veer et al., 2012), to confirm long-lasting effects of the drug. Finally, in a naïve cohort of mice, c-Fos immunohistochemistry was used to provide an index of neuronal activation (Kovacs, 1998) to examine the effects of JDTic on footshock-induced changes in the activity of stress-related brain areas.

Materials and Methods

Mice: Experiments were performed in 8-10-week-old male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME). Mice were housed 4 per cage upon arrival and allowed to acclimate to the vivarium for one week prior to testing. Mice were maintained on a

12:12-h light-dark cycle (0700h lights on) with *ad libitum* food and water available except during testing. Experiments were conducted during the light phase of the daily cycle to avoid startle ceiling effects (Chabot and Taylor, 1992). Experimental protocols were approved by the Institutional Animal Care and Use Committee of McLean Hospital and in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Academies Press, Washington D.C., USA, 2011).

Drugs: JDTic was synthesized at Research Triangle Institute (Research Triangle Park, NC). U50,488 was purchased from Sigma-Aldrich (St. Louis, MO). JDTic and U50,488 were dissolved in 0.9% saline and administered intraperitoneally (IP) at 10 mL/kg. The dose of U50,488 was based on its salt form.

Startle testing: The equipment and procedures used here were similar to those described in Chapter 2. Briefly, acoustic startle data were collected by measuring the amplitude of the startle reflex in response to white noise bursts of various intensities using the Med Associates Inc. (St. Albans, VT) Startle Reflex System and Advanced Startle software program. Mice were placed into 8.5 x 7 x 7 cm Plexiglas holders with steel rod floor bars attached to a load cell platform contained within a 40 x 64 x 42 cm sound-attenuating chamber. The load cell transduces movement into an electrical output that is amplified and digitized into arbitrary units by an analog-to-digital converter interfaced to a computer. Startle amplitude was defined as the maximum peak-to-peak voltage occurring within the first 100 ms after the onset of the startle stimulus. An audio stimulator generated 50 msec startle stimuli (1-32 kHz white noise, 1 msec rise-decay) which were delivered through high-frequency speakers located 4 cm behind the cages.

The intensities of the startle stimuli were calibrated before use using customized software. All tests were conducted in darkness.

Prior work demonstrates that mild footshock can potentiate the acoustic startle reflex in rodents (Davis, 1989; Risbrough et al., 2009). To determine if KORs are involved in the footshock-potentiated startle phenomenon, mice were matched into groups with similar levels of baseline startle and were treated with the KOR antagonist JDTic (10 or 30 mg/kg, IP) or vehicle 24 hr prior to testing. The footshock-potentiated startle test procedure was adapted from Risbrough et al. (2009). Mice were initially given a baseline startle session consisting of a 5 min acclimation period followed by 102 startle stimuli, 34 each at 80, 90, and 100 dB. This test served to habituate the mice to both the holding chambers and startle stimuli and to match them into experimental groups with equivalent baselines before testing. During the test session, mice received a 5 min acclimation period followed by 27 startle stimuli, 9 each at 80, 90, and 100 dB in a pseudo-random order with a 15 sec ISI to assess their baseline startle (startle test). Mice then received three sessions of 5 footshocks (shock session). The footshock intensity increased between sessions (i.e., 0.2 mA, session 1; 0.4 mA, session 2; and 0.8 mA, session 3). The average ISI between footshocks was 60 sec (30-90 sec). Mice received an additional startle test after each shock session to assess the magnitude of startle potentiation elicited by footshock exposure. Fecal boli were also recorded at the end of the test session, as an index of somatic stress (Hall, 1934; Flint et al., 1995; Crawley, 2007). The following day, mice were given a final startle test to probe context conditioning in the absence of footshock; fecal boli were again counted at the end of the

session. Mouse holders were thoroughly cleaned with 70% isopropanol wipes at the end of each test session.

Warm water tail-flick assay: The tail-flick assay was used to assess KOR-mediated analgesia (Vaught and Takemori, 1979; McLaughlin et al., 2003a; Van't Veer et al., 2012). The test was conducted 24 hr following the final startle test session. Mice were scruffed with their tail free and back supported and approximately 1 cm of their tail was submerged into a 52 ± 1°C warm water bath. The latency for mice to withdraw their tail from the water was timed with a stopwatch. A maximum time of 15 sec was imposed to prevent damage to the submerged tail. After taking baseline latencies, mice were treated with the KOR agonist U50,488 (15 mg/kg, IP), and withdrawal latencies were assessed 30 min later.

C-Fos immunohistochemistry: Separate cohorts of mice were used for c-Fos immunohistochemistry. Mice were placed in the startle chambers for 30 min on four consecutive days prior to testing to habituate them to the experimental procedure and reduce handling-induced c-Fos expression. On the fourth day, mice received JDTic (30 mg/kg, IP) or vehicle following the habituation session, immediately before being placed back into their home cage. Mice were tested 24 hr after injection. The test was designed to mimic the first two footshock blocks (0.2 mA and 0.4 mA) received in the footshock-potentiated startle test described above, but without the startle stimuli, in order to isolate the effects shock. Briefly, during the 30 min test session, 5 footshocks at 0.2 mA were presented 12.5 min after mice were placed in the chambers, followed 8.5 min later by 5 footshocks at 0.4 mA. Mice were returned to their home cages and,

120 min later, perfused with 4% paraformaldehyde. The procedures used for c-Fos immunolabeling were identical to those described in Chapter 2. Briefly, brains were postfixed in 4% paraformaldehyde overnight, then transferred into 30% sucrose in PBS for cryoprotection. Mouse brain sections (40 µm) were first incubated for 30 min in 0.3% H_2O_2, and then in a blocking solution of 5% NGS (Vector Laboratories, Inc., Burlingame, CA) for 1 hr. Sections were then incubated overnight with anti-c-Fos (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 5% NGS. The following morning, sections were first incubated for 1 hr in biotinylated anti-rabbit IgG (1:1,000; Vector Laboratories), and then in ABC for 1 hour (ABC Elite Kit, Vector Laboratories). C-Fos immunoreactivity was visualized with DAB. Photomicrographs of regions of interest were obtained at 40x on a Zeiss Axio Scope (Carl Zeiss Inc., Thornwood, NY) using OpenLab software (PerkinElmer, Waltham, MA). The number of labeled cells for each photomicrograph was counted using ImageJ software (NIH, Bethesda, MD) and the counts from 3 non-serial sections per region were averaged for each mouse

Data Analysis: Baseline startle was analyzed with a JDTic x dB level ANOVA with repeated measures (dB level). The effects of JDTic on footshock-potentiated startle were analyzed with a 3-way (JDTic x dB level x startle block) ANOVA with repeated measures (dB level and startle block). For the tail flick assay, a 3-way (JDTic x shock x U50,488) ANOVA with repeated measures (U50,488) was used to examine whether JDTic affected U50,488-induced antinociception. Footshock reactivity was analyzed by a 3-way (JDTic x shock x shock intensity) ANOVA with repeated measures (shock intensity). Fecal boli and c-Fos cell counts were analyzed with 2-way (JDTic x shock) ANOVAs or t-tests as appropriate. Significant interactions in the ANOVAs were further

analyzed using Bonferroni post tests. Data are graphed as mean plus standard error of the mean (SEM).

Results

Because of the pharmacodynamics of JDTic, mice received the drug (10 mg/kg, IP) or vehicle 24 hr before footshock-potentiated startle testing. Vehicle and JDTic-treated mice did not significantly differ in their preshock baseline startle reactivity across the three decibel levels (80, 90 and 100) tested ($F[1,54]=0.015$, NS; **Table 3.1**). Startle amplitude increased as a function of dB level as demonstrated by a main effect of dB ($F[2,108]=196.04$, $P<0.001$). Following the baseline startle block, mice received 3 footshock blocks ascending in amplitude (0.2, 0.4 and 0.8 mA) each followed by a startle test. The startle reactivity following each footshock block was compared to the baseline for each mouse to calculate footshock potentiation. Vehicle- and JDTic-treated mice that did not receive footshock were also tested to confirm that JDTic had no delayed effects on startle reactivity (**Table 3.2**). Analysis of shocked mice revealed a dB x JDTic interaction ($F[2,128]=3.24$, $P<0.05$; **Fig. 3.1A**). There was a trend for JDTic to decrease startle potentiation at 80 dB ($t[32]=1.68$, $P=0.10$), whereas 90 and 100 dB were not significantly affected. JDTic effects were not due to differences in detecting the footshock, since reactivity during footshock presentation did not differ between the groups (**Table 3.3**). The number of fecal boli also did not differ between shocked groups (i.e., vehicle- and JDTic-treated), but was significantly increased in shocked mice compared to unshocked controls ($F[1,54]=33.33$, $P<0.0001$; **Fig 3.1B**). One day following footshock-potentiated startle testing, mice were returned to the test chamber and given another startle test in the absence of footshock. In this test, JDTic did not

affect startle magnitude compared to controls (dB x JDTic, F[2,64]=2.51, NS; data not shown). The ability of the KOR agonist U50,488 to induce analgesia was tested the following day to ensure persistent JDTic antagonism of KORs (**Fig. 3.1C**). Tail withdrawal latencies depended upon a significant JDTic x U50,488 interaction (F[1,54]=7.30, $P<0.01$). Between-subject analysis indicated vehicle and JDTic mice did not differ at baseline, but were significantly different following U50,488 ($P<0.01$). Surprisingly, within-subject effects indicated a significant increase in tail withdrawal latency following U50,488 in both vehicle ($P<0.001$) and JDTic ($P<0.05$) treated mice, although the antinociceptive effects of U50,488 were clearly reduced by JDTic treatment.

Table 3.1 Baseline startle reactivity prior to footshock

	JDTic (10 mg/kg)			
dB	**Veh no shock**	**JDTic no shock**	**Veh shock**	**JDTic shock**
80	1.23 ± 0.14	1.31 ± 0.15	1.06 ± 0.12	1.28 ± 0.18
90	2.33 ± 0.42	2.54 ± 0.25	2.58 ± 0.29	2.58 ± 0.35
100	4.00 ± 0.43	3.65 ± 0.34	4.35 ± 0.35	3.99 ± 0.38

	JDTic (30 mg/kg)			
dB	**Veh no shock**	**JDTic no shock**	**Veh shock**	**JDTic shock**
80	0.80 ± 0.10	0.64 ± 0.04	0.69 ± 0.07	0.76 ± 0.06
90	1.70 ± 0.17	1.69 ± 0.21	1.55 ± 0.14	1.85 ± 0.21
100	3.42 ± 0.27	3.42 ± 0.34	3.39 ± 0.25	3.29 ± 0.27

Measures reported as mean ± SEM. No significant effects of JDTic or shock group were observed.

Table 3.2 Percent change from baseline in mice that did not receive footshock

		JDTic (10 mg/kg)		JDTic (30 mg/kg)	
Block	**dB**	**Veh**	**JDTic**	**Veh**	**JDTic**
1	80	-21.16 ± 8.23	-15.14 ± 6.97	-0.42 ± 11.54	-5.52 ± 4.89
	90	5.77 ± 9.86	-9.70 ± 7.01	-16.11 ± 8.13	-13.21 ± 6.91
	100	-6.95 ± 9.98	6.31 ± 13.56	-1.53 ± 8.32	-18.61 ± 7.15
2	80	-22.74 ± 7.66	-23.51 ± 11.72	-15.37 ± 8.99	-18.63 ± 6.22
	90	0.17 ± 12.92	-21.34 ± 6.66	-9.75 ± 12.41	-16.49 ± 7.53
	100	-1.93 ± 6.98	-4.68 ± 9.12	-8.5 ± 7.34	-16.83 ± 8.43
3	80	-29.41 ± 8.32	-29.91 ± 7.57	-22.79 ± 8.07	-21.69 ± 4.83
	90	3.36 ± 12.08	-23.90 ± 7.92	-11.2 ± 10.35	-11.09 ± 10.12
	100	-9.70 ± 6.34	-0.26 ± 10.86	-15.83 ± 6.11	-5.59 ± 9.77

Block refers to the startle session following test period where shocked mice received 0.2 mA footshock (Block 1), 0.4 mA (Block 2) and 0.8 mA (Block 3).

Figure 3.1 Footshock-potentiated startle in mice treated with 10 mg/kg JDTic. Mice were matched into groups with equivalent baseline startle and then administered JDTic (10 mg/kg) 24 hr before testing. ***A.*** Acoustic startle reactivity to 80, 90, and 100 dB stimuli was tested following blocks of ascending footshock (0.2, 0.4, and 0.8 mA) and compared to baseline preshock levels to calculate potentiation. Analysis revealed a dB x JDTic interaction ($P<0.05$). JDTic reduced footshock-potentiated startle at 80 dB although this did not reach significance ($P=0.10$). ***B.*** Shocked mice produced more fecal boli compared to no-shock control mice. JDTic did not affect fecal boli during the test session. ***C.*** Following testing, the warm water tail-flick assay was used to assess KOR-mediated analgesia. The latency for mice to withdraw their tail from a 52°C water bath at baseline and 30 min after administration of the KOR agonist U50,488 (15 mg/kg, IP) was timed with a stopwatch. U50,488 increased tail withdrawal latencies in both vehicle (Veh) and JDTic (10 mg/kg) treated mice. Tail withdrawal latencies following U50,488 were lower in JDTic mice compared to Veh. Data were analyzed by ANOVA followed by Bonferroni post test as appropriate. * $P<0.05$, *** $P<0.001$, **** $P<0.0001$ between group comparisons ^^ $P<0.01$ within group comparison

Figure 3.1 (Continued) Footshock-potentiated startle in mice treated with 10 mg/kg JDTic

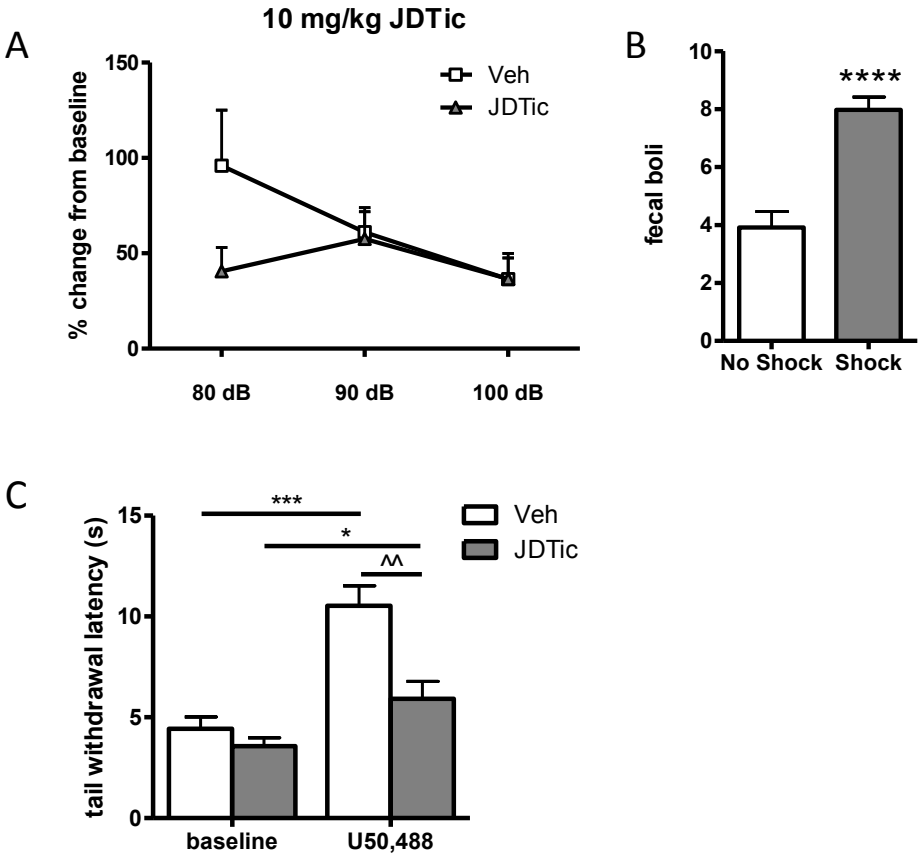


Table 3.3 Footshock reactivity in JDTic-treated mice

	JDTic (10 mg/kg)			
	no shock		shock	
mA	Veh	JDTic	Veh	JDTic
0.2	0.35 ± 0.02	0.43 ± 0.07	5.65 ± 0.84	4.85 ± 0.60
0.4	0.49 ± 0.10	0.73 ± 0.27	11.95 ± 0.85	10.81 ± 0.94
0.8	0.57 ± 0.18	0.48 ± 0.14	11.55 ± 0.85	10.71 ± 0.85

	JDTic (30 mg/kg)			
	no shock		shock	
mA	Veh	JDTic	Veh	JDTic
0.2	0.27 ± 0.03	0.29 ± 0.03	4.69 ± 0.58	5.40 ± 0.51
0.4	0.25 ± 0.02	0.26 ± 0.02	11.17 ± 0.66	11.08 ± 0.81
0.8	0.23 ± 0.02	0.21 ± 0.02	11.33 ± 0.61	11.30 ± 0.88

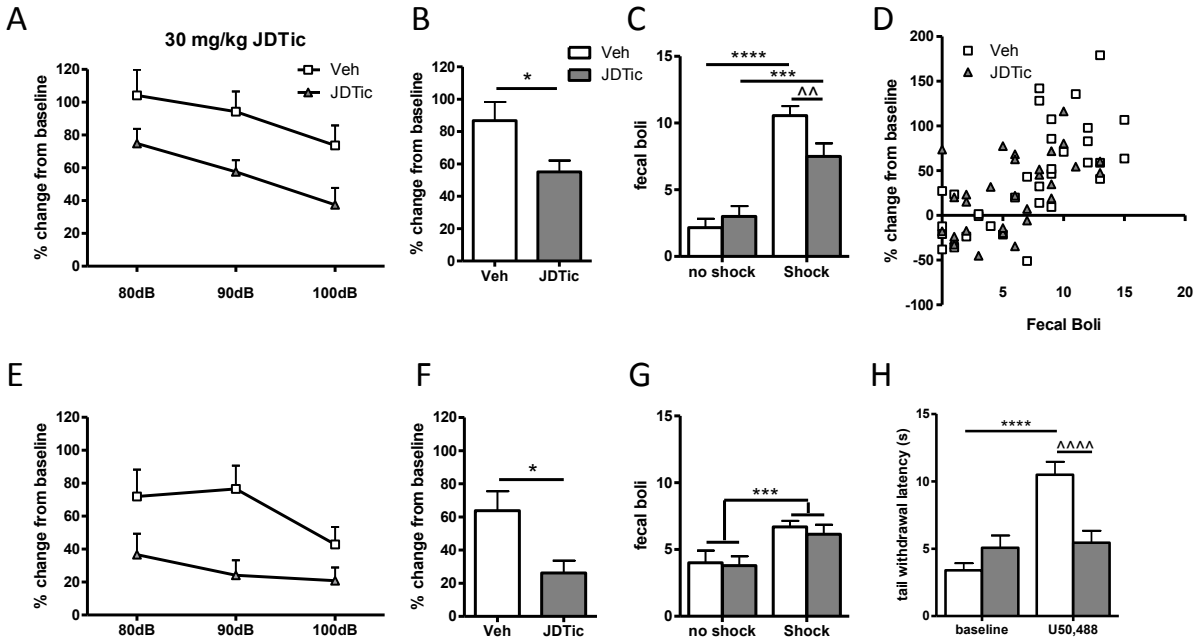
Measures reported as mean ± SEM of average reactivity during the presentation of footshock. No significant effects of JDTic were observed.

Previously we have shown dose effects of JDTic in blocking another stress-induced behavior: CRF-enhanced startle (Chapter 2). In the present studies, we observed a small but significant increase in tail withdrawal latency in JDTic pretreated mice given the KOR agonist U50,488; these data, combined with data from Chapter 2 indicating that 10 mg/kg JDTic did not block CRF-induced startle, suggest that 10 mg/kg is not a sufficient dose to fully antagonize KOR function in mice. We therefore repeated the footshock-potentiated startle test in a separate cohort of mice that received JDTic at 30 mg/kg, IP. JDTic treatment did not have an effect on baseline startle amplitude ($F[1,69]=0.026$, NS; **Table 3.1**). Analysis of test data from the shocked mice demonstrated main effects of startle block ($F[2,74]=12.39$, $P<0.001$), dB ($F[2,74]=4.67$, $P<0.05$) and JDTic ($F[1,37]=5.29$, $P<0.05$) but no interactions (**Fig. 3.2A)**. Mice pretreated with JDTic before testing had overall lower levels of startle when collapsed across dB level and startle block ($P<0.05$; **Fig. 3.2B**). Footshock increased the number of fecal boli during the test session compared to no-shock controls (main effect of footshock, $F[1,59]=72.75$, $P<0.0001$; **Fig. 3.2C**) and there was an interaction of JDTic with footshock ($F[1,59]=6.22$, $P<0.05$). While JDTic did not affect the number of fecal boli produced by unshocked mice, it significantly reduced footshock-induced increases ($P<0.05$; **Fig. 3.2C**). Further, the number of fecal boli strongly correlated with potentiated startle averaged across dB and startle block ($r[63]=0.65$, $P<0.0001$; **Fig. 3.2D**). During the context test on the following day, JDTic-treated mice continued to show decreased startle amplitude compared to controls (main effect of JDTic, $F[1,37]=4.36$, $P<0.05$; **Fig. 3.2E&F**) regardless of dB level. There was also a main effect of dB ($F[2,37]=4.36$, $P<0.05$), with less potentiation seen with increasing dB level in both treatment groups (**Fig. 3.2E**). The number of fecal boli counted after the context

test session did not differ between shocked groups, but was significantly increased over no-shock control mice ($F[1,46]=13.37$, $P<0.001$; **Fig. 3.2G**). In the tail-flick, U50,488 effects depended on an interaction with JDTic ($F[1,76]=479.83$, $P<0.0001$). Within subjects analysis indicated a significant increase in tail withdrawal latency following U50,488 treatment in vehicle ($P<0.0001$) but not JDTic-treated mice (**Fig 3.2H**). There were no differences between drug treatment groups at baseline, but a significant increase in tail withdrawal latency in vehicle compared to JDTic following U50,488 ($P<0.0001$).

Figure 3.2 Footshock-potentiated startle in mice treated with 30 mg/kg JDTic. ***A.*** JDTic treatment significantly reduced footshock-potentiated at all dB levels. ***B.*** Data collapsed across block and dB revealed a main effect of JDTic. ***C.*** Footshock significantly increased fecal boli compared to no-shock controls. In shocked mice, JDTic significantly reduced the number of fecal boli counted at the end of the test session. ***D.*** Average startle potentiation during the test session significantly correlated with fecal boli ($P<0.0001$). ***E.*** JDTic continued to reduce startle reactivity when mice were returned to the footshock test chamber 24 hr following testing as revealed by a main effect of JDTic treatment (***F.***). ***G.*** While fecal boli numbers were increased in shocked mice compared to controls, JDTic did not affect this measure during the re-test. ***H.*** Veh mice showed an increase in tail withdrawal latencies following U50,488, which was absent in JDTic (30 mg/kg) treated mice. Data were analyzed by ANOVA followed by Bonferroni post test as appropriate. * $P<0.05$, *** $P<0.001$, **** $P<0.0001$ between group comparisons, ^^ $P<0.01$, ^^^^ $P<0.0001$ within group comparisons

Figure 3.2 (Continued) Footshock-potentiated startle in mice treated with 30 mg/kg JDTic



C-Fos immunoreactivity was quantified as an index of neuronal activation to determine the effects of JDTic on footshock-induced brain circuit activation. Mice received 2 sets of footshocks (5 each at 0.2 mA and 0.4 mA) across a 30 min test session following the same time course as that used in the footshock-potentiated startle paradigm. The session was terminated after 0.4 mA shocks in order to capture the peak effect of JDTic on anxiety-like behavior. Mice receiving footshock had higher reactivity during footshock presentation compared to activity during the same test period in no-shock controls. JDTic did not affect this measure (footshock amplitude x shock treatment interaction, $F[1,32]=29.46$, $P<0.0001$; main effect of footshock amplitude, $F[1,32]=26.22$, $P<0.0001$; main effect of footshock, $F[1,32]=304.41$, $P<0.0001$; **Table 3.4**). To verify the ability to detect increases in c-Fos labeling following footshock, c-Fos cell counts were made in the PVN, a region highly responsive to stress. In vehicle-pretreated mice, footshock induced a significant increase in c-Fos cell counts of this region compared to no-shock controls (**Fig. 3.3**). Subsequent counts were made in the VTA and CA1, CA2, CA3 and dDG subregions of the HIP (**Fig. 3.4**). Neither footshock nor JDTic had an effect on c-Fos immunoreactivity in these brain regions (**Table 3.5**).

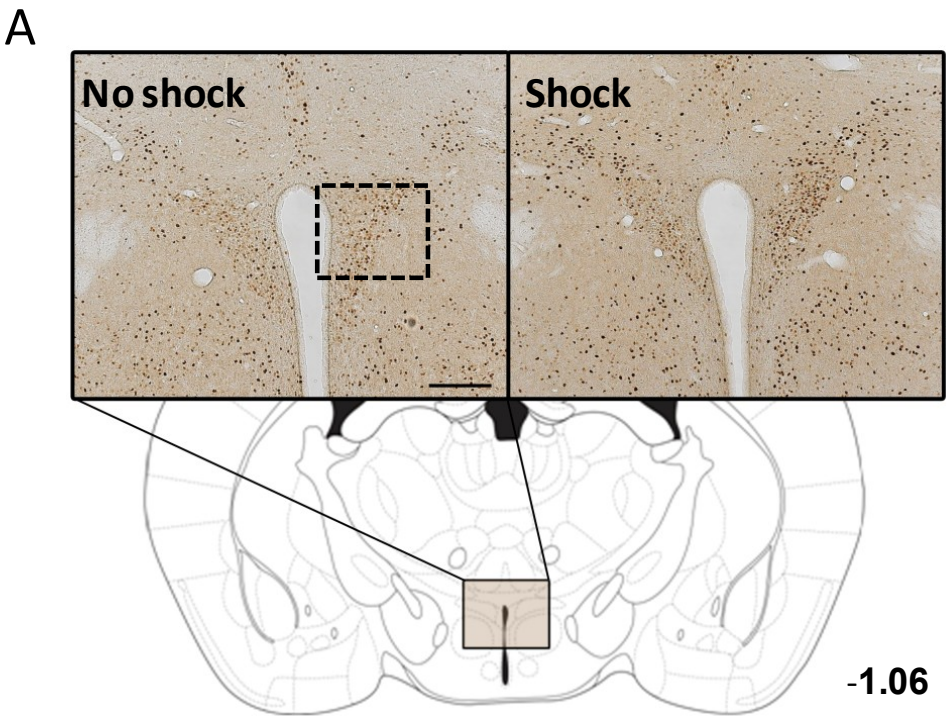
Table 3.4 Footshock reactivity in JDTic-treated mice used for c-Fos studies

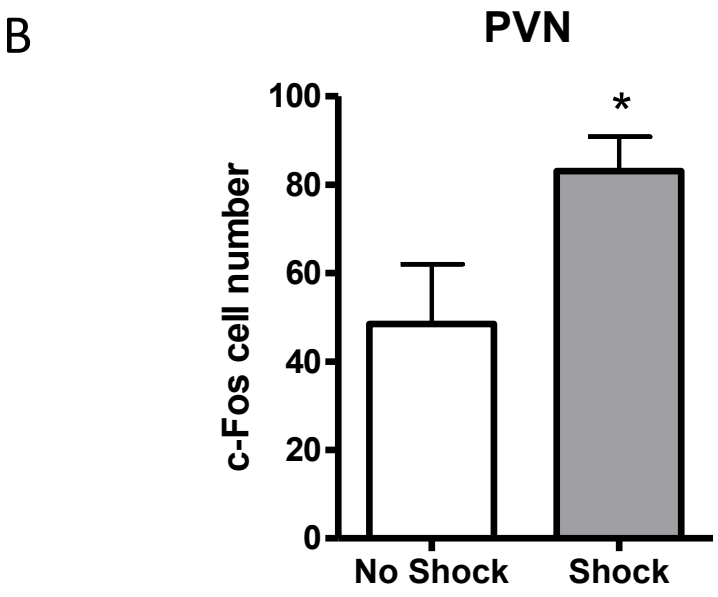
	No shock		Shock	
mA	**Veh**	**JDTic**	**Veh**	**JDTic**
0.2	0.56 ± 1.40	0.53 ± 0.05	9.18 ± 1.19	7.58 ± 0.69
0.4	0.46 ± 0.06	0.37 ± 0.06	13.68 ± 1.02	12.22 ± 0.63

Measures reported as mean ± SEM of average reactivity during the presentation of footshock. No significant effects of JDTic were observed.

Figure 3.3 Effect of footshock on c-Fos immunoreactivity in the PVN. ***A.*** Representative photomicrographs of c-Fos staining in the PVN at 10x in an unshocked (left) and shocked (right) mouse. Scale bar indicates 200 μm. Dashed box is representative of area counted at 40x for analysis. Anteroposterior distance from Bregma is indicated at right. ***B.*** Footshock significantly increased c-Fos counts in the PVN. Data were analyzed by t-test. *$P<0.05$. PVN, paraventricular nucleus of the hypothalamus

Figure 3.3 (Continued) Effect of footshock on c-Fos immunoreactivity in the PVN





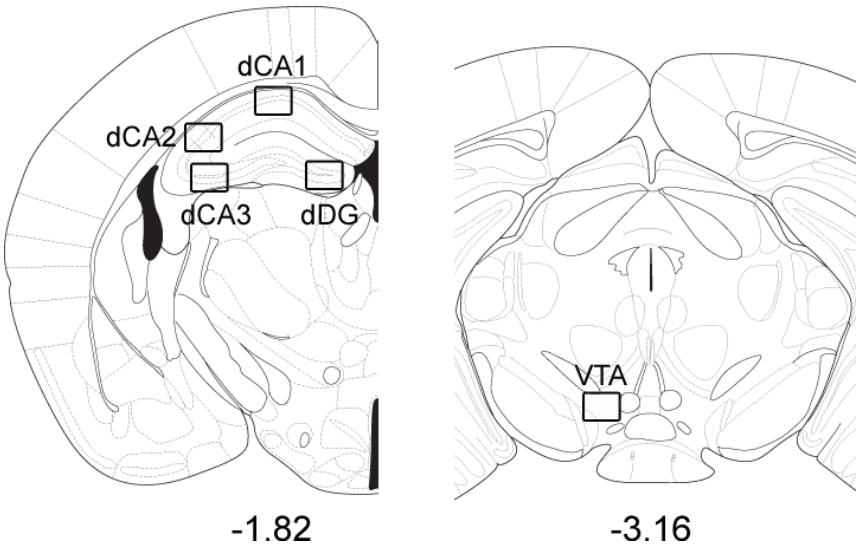


Figure 3.4 Location of regions of interest analyzed for c-Fos immunoreactivity. Areas are demarcated by boxes on representative atlas images of the mouse brain (figures 46 and 57 in Paxinos and Franklin (2001)). Anteroposterior distance from Bregma is indicated below each image. The definitions of abbreviations used are located in **Table 3.5**.

Table 3.5 Effect of JDTic on c-Fos expression after footshock

Brain Area	Sub-division	Abbr.	c-Fos Cell # (mean ± SEM)				*P*-value*
		IP condition	Saline no shock	JDTic no shock	Saline shock	JDTic shock	
Dorsal hippocampus	field CA1	dCA1	13.1 ± 3.9	11.1 ± 2.4	9.6 ± 2.5	8.4 ± 1.3	0.89
	field CA2	dCA2	19.8 ± 3.2	15.5 ± 2.4	14.9 ± 2.6	13.6 ± 1.8	0.55
	field CA3	dCA3	17.5 ± 3.1	17.7 ± 2.2	14.8 ± 1.8	14.8 ± 1.1	0.94
	dentate gyrus	dDG	21.5 ± 0.9	21.6 ± 1.6	19.9 ± 1.1	21.9 ± 2.0	0.56
Ventral Tegmental Area		VTA	10.0 ± 1.2	11.0 ± 0.9	9.5 ± 1.0	11.5 ± 1.7	0.72

*p-value for JDTic x CRF interaction

n=8-10 per group

Discussion

The current study examined the role of KORs in footshock-potentiated startle, a model of contextual fear learning. We show that pretreatment with a single injection of JDTic, a highly selective KOR antagonist with long-lasting effects (Carroll et al., 2004), can reduce footshock effects on startle. These effects were mild in mice treated with IP injections of 10 mg/kg JDTic; the largest difference was observed at the lowest startle intensity tested (80 dB) and did not reach statistical significance. A higher dose of JDTic (30 mg/kg) significantly attenuated startle responding following footshock across all dB levels and footshock intensities. Neither dose of JDTic affected baseline startle responding or footshock reactivity, indicating specific effects of KOR antagonism on stress-induced anxiogenic-like behavior. These data confirm a role for KORs in increases in startle produced by stress.

Footshock reactivity is here defined as the magnitude of the "flinch" response, which causes a downward displacement of the load cell beneath the holding cage, during footshock delivery. Prior treatment with JDTic did not affect this measure, suggesting both vehicle and JDTic mice experienced the aversive quality of the footshock similarly. This may seem surprising since JDTic reportedly blocks stress-induced analgesia (McLaughlin et al., 2006b). However, footshock-induced analgesia specifically is usually obtained using higher shock intensities of longer duration over a more extensive shock session (Lewis et al., 1980; Lewis et al., 1983; Menendez et al., 1993b). Thus the shock duration, amplitude, and interval between footshock presentations in the present study may not have been effective at inducing opioid-mediated analgesia (Ross and Randich, 1984). Considering that vehicle and JDTic-treated mice did not

significantly differ in footshock responsiveness, JDTic-induced decreases in potentiation cannot be attributed to decreased sensitivity to footshock.

Conceivably, JDTic could produce an apparent anxiolytic-like effect on startle if the drug instead increased fear-like states since footshock can produce an inverted U-shaped response on startle amplitude (Davis and Astrachan, 1978) as animals begin to freeze, a behavior that is incompatible with startle. We do not believe this to be the case considering experiments in which JDTic decreased footshock-potentiated startle, it also significantly decreased the number of fecal boli. If instead this reduction in startle were due to increased anxiety-like behavior, an increase in defecation should have been observed. Because fecal boli correlated with average startle potentiation, these data suggest that decreased startle reactivity is indicative of a reduced anxiety-like state.

The ability of JDTic to reduce footshock effects was not due to a general decrease in startle responsiveness since JDTic alone had no effects on baseline startle. This is in agreement with previous work using these JDTic doses in mice (Chapter 2) as well as JDTic and norBNI studies in rat (Bortolato et al., 2005; Knoll et al., 2007; Tejeda et al., 2010a). Potentiation of startle following footshock was calculated as the percent change in startle amplitude from baseline levels collected at the start of the test session. The ASR is an involuntary brainstem reflex that can be modulated by limbic input based on affective state (Lang et al., 1990). Thus aversive stimuli can enhance, whereas pleasant stimuli can attenuate, the ASR (Vrana et al., 1988). The data presented here suggest that KOR antagonist treatment does not induce anxiolytic-like effects in non-stressed animals as measured by the ASR, since JDTic and vehicle mice had

equivalent levels of baseline startle. Instead, JDTic specifically augmented increases in startle following an aversive stimulus, indicated by the inability of footshock to increase startle up to control levels in JDTic-treated mice. To induce KOR blockade, mice were treated with JDTic 24 hr before the test session, during which potentiation of the ASR was elicited immediately after footshock presentation. Because KORs were antagonized before footshock presentation, this experimental design did not allow us to distinguish between KOR blockade prior to and following the association of context and footshock. Future studies implementing different KOR treatment times would enable us to determine the role of KORs in acquisition, consolidation, and expression of context-dependent potentiation of startle. For instance, JDTic could be administered subsequent to footshock and prior to follow-up context testing in order to investigate expression directly. Previous work has demonstrated that KOR blockade is effective at decreasing stress-induced deficits in novel object recognition when given immediately after stress (Carey et al., 2009). Based on this, would expect reductions in context-elicited potentiation of startle if JDTic was administered immediately after the test session. Further work on the effects of KORs during specific phases of the test session will clarify whether KOR antagonists would be beneficial not only when stress can be predicted, but in cases when stress has already occurred.

Previous work has shown that footshock induces a robust increase in c-Fos activation in the PVN (Pezzone et al., 1992; Duncan et al., 1996; Li and Sawchenko, 1998). Here, a significant increase in c-Fos cell counts was also observed following footshock in the PVN of vehicle-treated mice compared to no-shock controls. However, this induction was not as substantial as others noted in the literature. Mice were killed 2 hours after a

30 min footshock paradigm designed to mimic part of the footshock-potentiated startle protocol. This time point is consistent with numerous studies indicating optimal detection of c-Fos 2 hr following an aversive stimulus (Morgan and Curran, 1991), suggesting that the time point of tissue collection was not responsible for the minimal effects observed. However, although other studies have collected tissue at a similar time point, their shock procedures were longer or more intense, which may result in greater c-Fos activation. The small, statistically significant effects in the present study may also be attributed to an increased number of c-Fos positive cells in no-shock control mice. In previous reports, baseline (i.e., no-shock) c-Fos reactivity is minimal in the PVN (Li and Sawchenko, 1998; Kwon et al., 2008). The reasons for this discrepancy are not clear. One possibility is that the mice were housed outside the testing room where footshock occurred; under these conditions, it is possible that unshocked mice became stressed upon hearing vocalizations of other mice receiving footshock, or remaining housed near cages of stressed mice before they were killed for the immunohistochemistry studies. Psychological stress, induced by having a mouse witness another mouse receive footshock, has not been shown to induce c-Fos expression in the PVN (Kwon et al., 2008), although it is unclear whether these psychologically stressed mice had any sensory contact with mice that received footshock following the procedure. There is recent evidence that even indirect contact with stress-related cues can cause depressive- or anxiety like behavior in mice (Warren et al., 2013), although it remains unclear whether the critical cues are visual, auditory, or olfactory. Regardless, no differences between shocked and unshocked mice were seen in any region of the HIP or the VTA, regions in which c-Fos induction following footshock have been shown (Smith et al., 1992; Funk et al., 2003; Funk et al., 2006).

The elevated baseline c-Fos levels in the PVN of control mice may correspond with higher levels of c-Fos cell counts in other stress responsive regions; therefore, subtle increases in c-Fos reactivity might have been too small to detect. Considering these limitations, it is difficult to ascertain whether JDTic truly lacked effect on brain activation as measured by c-Fos immunoreactivity, or whether changes were obscured as a result of signal-detection problems related to the design or implementation of our studies. This question might be resolved in the future using high-power (9.4 Tesla) functional imaging techniques—currently under development at McLean Hospital—to investigate circuit activation in JDTic-treated mice receiving footshock during brain imaging.

Although we were unable to detect changes in neuronal activation using c-Fos labeling in brain regions such as the HIP and VTA following footshock, there are obvious candidate brain regions where KOR activation may be affecting footshock-induced anxiety-like behavior. We focused on the HIP and VTA here because we have previously identified JDTic-mediated reductions in CRF-induced c-Fos immunoreactivity in the dDG and VTA (Chapter 2), which may also be key regions for JDTic effects on footshock-potentiated startle, given the critical role of CRF in this response (Risbrough et al., 2009). In addition, the HIP has been specifically implicated in contextual fear conditioning using paradigms similar to those used here (Kim and Fanselow, 1992; Phillips and LeDoux, 1992; Anagnostaras et al., 1999; Rudy et al., 2002). However, KORs are also highly expressed in the AMY (Mansour et al., 1995) a region critical for footshock-potentiated startle (Hitchcock et al., 1989) which sends direct projections to the startle pathway (Inagaki et al., 1983; Rosen et al., 1991). Microinfusions of KOR antagonist into the AMY have anxiolytic-like effects in models of conditioned and

unconditioned fear (Knoll et al., 2011), and abolish stress-induced increases in anxiety-like behavior (Bruchas et al., 2009). In addition, KORs are expressed on terminal inputs to the BNST (Li et al., 2012), another brain region often implicated in anxiety (Radke, 2009; Davis et al., 2010). The AMY and BNST represent candidate brain regions for future studies of c-Fos in the context of footshock-potentiated startle.

The stress response is part of an adaptive mechanism responsible for maintaining homeostasis in response to a real or perceived threat; however, intense or sustained stress contributes to the pathophysiology of psychiatric disorders. The present work confirms a role for KORs in stress-induced behavior by demonstrating that KOR antagonism significantly attenuates footshock-induced increases in context-dependent startle reactivity. This anxiolytic-like profile, in combination with previous work demonstrating anti-depressant-like effects of KOR blockade (Newton et al., 2002; Mague et al., 2003; Shirayama et al., 2004; Carr et al., 2010), support the development of therapeutics targeting the KOR system, particularly in treating comorbid anxiety and depressive disorders. Nevertheless, improved treatments may emerge from a deeper understanding of the brain regions and cell types involved in these anxiolytic- and antidepressant-like effects. A limitation of the current study was the use of systemic KOR antagonist treatment, which precluded our ability to attribute JDTic effects to any particular brain region. In order to target specific KOR systems, we developed a line of floxed KOR mice that enable Cre recombinase (Cre)-induced ablation of KORs in specific brain regions or cell populations. The next chapter describes the generation and basic characterization of two mouse lines developed by breeding our floxed KOR mice with mouse lines expressing Cre in early embryogenesis (Ella-Cre) or only in DA

neurons (DAT-Cre) to produce mice lacking KORs throughout the brain and body or lacking KORs specifically in DA neurons, respectively.

Chapter 4

Ablation of kappa-opioid receptors from brain dopamine neurons has anxiolytic-like effects and enhances cocaine-induced plasticity

Ashlee Van't Veer*[1], Anita J. Bechtholt*[1], Sara Onvani[1], David Potter[1], Yujun Wang[2], Lee-Yuan Liu-Chen[2], Günther Schütz[3], Elena H. Chartoff[1], Uwe Rudolph[1], Bruce M. Cohen[1], and William A. Carlezon Jr.[1]

*Denotes equal authorship

[1]Department of Psychiatry, Harvard Medical School, McLean Hospital, Belmont, MA 02478; [2]Department of Pharmacology, Temple University School of Medicine, Philadelphia, PA, 19140; and [3]Division of Molecular Biology of the Cell I, German Cancer Research Center, 69120 Heidelberg, Germany

Author contributions: AVV performed the tail-flick, Cre-recombinase immunohistochemistry, hearing tests and cocaine-conditioned place preference. AVV and AJB performed the cocaine sensitization and visual cliff experiments. SO weighed the mice and helped maintained the mouse colony. DP performed the qPCR experiments. YW and LYLC designed and performed the binding studies and analysis. EHC provided technical assistance for qPCR studies and analyzed the qPCR data. GS

provided the DAT-Cre mice. UR designed the floxed mice and provided technical assistance. AVV, AJB, BC, and WAC designed the experiments.

Abstract

Brain kappa-opioid receptors (KORs) are implicated in states of motivation and emotion. KORs negatively regulate mesolimbic dopamine (DA) neurons, and agonists produce depressive-like behavioral effects. To further evaluate how KOR function affects behavior, we developed mutant mice in which exon 3 of the KOR gene (*Oprk1*) was flanked with Cre-lox recombination (loxP) sites. By breeding these mice with lines that express Cre-recombinase (Cre) in early embryogenesis (EIIa-Cre) or only in DA neurons (DAT-Cre), we developed constitutive KOR knockouts ($KOR^{-/-}$) and conditional knockouts that lack KORs in DA-containing neurons (DAT-$KOR^{lox/lox}$). Autoradiography demonstrated complete ablation of KOR binding in the $KOR^{-/-}$ mutants, and reduced binding in the DAT-$KOR^{lox/lox}$ mutants. Quantitative polymerase chain reaction (qPCR) studies confirmed that KOR mRNA was undetectable in the constitutive mutants and reduced in midbrain DA systems in the conditional mutants. Behavioral characterization demonstrated that these mutant lines do not differ from controls in metrics including hearing, vision, weight, and locomotor activity. Whereas $KOR^{-/-}$ mice appeared normal in the open field and light/dark box tests, DAT-$KOR^{lox/lox}$ mice showed reduced anxiety-like behavior, broadly consistent with previously reported effects of KOR antagonists. Sensitization to the locomotor-stimulating effects of cocaine appeared normal in $KOR^{-/-}$ mutants, but exaggerated in DAT-$KOR^{lox/lox}$ mutants. Exaggerated sensitivity to cocaine in the DAT-$KOR^{lox/lox}$ mutants is consistent with a role for KORs in negative regulation of DA function, whereas the lack of differences in the $KOR^{-/-}$ mutants suggests more thorough compensatory adaptations after constitutive receptor ablation. These mouse lines may be useful in future studies of KOR function.

Introduction

Accumulating evidence indicates that brain kappa-opioid receptors (KORs) and dynorphin (DYN), the endogenous ligand that binds at these receptors (Chavkin et al., 1982), are involved in regulating states of motivation and emotion. Administration of KOR agonists produces depressive (dysphoric) effects in humans (Pfeiffer et al., 1986) and depressive-like effects in rodents (for review, see Carlezon et al., 2009; Bruchas et al., 2010; Knoll and Carlezon, 2010). In contrast, KOR antagonists produce antidepressant-like effects (for review, see Carlezon et al., 2009; Carlezon and Carroll, in-press). Although the mechanisms through which KORs regulate mood are not fully understood, actions upon the mesocorticolimbic dopamine (DA) system appear to play a key role. KORs are expressed on both the cell bodies and terminals of mesocorticolimbic (ventral tegmental area [VTA]) DA neurons (Svingos et al., 1999; Svingos et al., 2001) and are coupled to G-proteins, such that agonist stimulation inhibits cyclic AMP production and modulates potassium and calcium channel conductance (Bruchas and Chavkin, 2010). These processes produce inhibition of DA neurons in the VTA (Margolis et al., 2003; Margolis et al., 2006b; Ford et al., 2007) and diminished DA release in areas that receive VTA input such as the nucleus accumbens (NAc) and prefrontal cortex (PFC), regardless of whether the agonist is given systemically (Di Chiara and Imperato, 1988; Devine et al., 1993; Carlezon et al., 2006), into the VTA (Margolis et al., 2006b), or into the NAc (Donzanti et al., 1992). Systemic administration of KOR agonists also attenuates cocaine effects on behavior (Tomasiewicz et al., 2008) and DA neurochemistry (Maisonneuve et al., 1994; Thompson et al., 2000). KOR antagonists cause small elevations in extracellular concentrations of DA in the NAc (Maisonneuve et al., 1994), consistent with a

neuromodulatory role of KORs and DYN. Collectively, these studies suggest that manipulations that target KORs may be useful in the study and treatment of debilitating disorders characterized by dysregulation of motivation and emotion, such as mood disorders and addiction.

There is increasing interest in the development of KOR targeted ligands as therapeutic agents. It has been suggested that KOR antagonists might have a wide range of indications, including the treatment of depressive-, anxiety-, and addictive disorders (for review, see Carlezon et al., 2009; Wee and Koob, 2010; Tejeda et al., 2012; Carlezon and Carroll, in-press). A general ability to reduce the impact of stress may explain how KOR antagonists can have efficacy in such a wide variety of animal models that would appear to represent different disease states (Bruchas et al., 2010; Knoll and Carlezon, 2010; Van't Veer et al., 2012; Carlezon and Carroll, in-press). It has been suggested that partial KOR agonists, which activate KORs with a lower efficacy than DYN and thus may lack the dysphoric effects produced by full agonists, could be useful for the treatment of conditions characterized by elevated motivation (Carlezon et al., 2009). One such condition is mania (Cohen and Murphy, 2008), a hallmark sign of bipolar disorder. Full KOR agonists have long been of interest as non-addictive analgesic drugs (Pasternak, 1980) or anti-itch medications (Inan and Cowan, 2004). Although current full KOR agonists produce dysphoric effects that may render them difficult for patients to tolerate, recent work suggests that it may be possible to identify new generations of these agents that have the desired effects on pain without aversive side effects (Bruchas et al., 2011). As such, a broader understanding of the neurobiology of KOR function may facilitate the development of new and improved medications.

One strategy to understand the neurobiological significance of KORs is to develop mutant mice that lack these receptors. We generated a line of mice in which exon 3 of the KOR gene (opioid receptor, kappa 1 [*Oprk1*]) was targeted for deletion by flanking it with Cre-lox (loxP) recombination sites, thereby enabling conditional (region-specific) KOR deletion and allowing the design of new studies that complement work conducted in previously reported lines (Chefer et al., 2005). By breeding these mice with lines that express Cre-recombinase (Cre) in early embryogenesis (EIIa-Cre) or only in DA neurons (dopamine transporter [DAT]-Cre), we developed constitutive KOR knockouts ($KOR^{-/-}$) and conditional knockouts that lack KORs in DA-containing neurons (DAT-$KOR^{lox/lox}$). Here we describe initial characterization of these mice in molecular assays to confirm the efficacy of the mutation, as well as in a battery of behavioral tests that quantify effects of the mutations on metrics including hearing, vision, weight, locomotor activity in an open field and anxiety. We also examined the effects of acute and repeated cocaine on behavior in these mice, for comparison with findings from previous reports (Chefer et al., 2005). These early studies provide a basis for future studies of KOR function in these mice.

Materials and methods

Mice: Mice were group-housed (2-5 mice/cage) and maintained on a 12:12-h light-dark cycle (0700h lights on) with *ad libitum* food and water available except during behavioral testing. Experiments were conducted in male mice 2 to 4 months at the start of each experiment and testing occurred during the light phase of the daily cycle. Mice tested in the light/dark box test were also tested 7 d later in the elevated plus maze. For all of the other individual experiments, naïve (previously untested) mice were used. Some

additional tests were performed on the DAT-KOR$^{lox/lox}$ mice and appropriate controls to follow up positive findings in initial screening procedures (see below). Experimental protocols were approved by the Institutional Animal Care and Use Committee of McLean Hospital and in accordance with the National Institutes of Health (NIH) policies.

Development of mutant mice: The murine KOR gene (*Oprk1*) contains 4 exons. A targeting construct was generated in which loxP sites flanked exon 3 of this gene (inGenious Targeting Laboratory [iTL], Ronkonkoma, NY). Protein lacking this region would be non-functional as the excision shifts the open reading frame, which ensures that subsequent portions of the protein are not translated. For positive selection of homologous recombinants, an FRT-flanked neomycin resistance gene (neo) was cloned downstream of exon 3 (**Fig. 4.1**). The targeting vector was linearized and transfected by electroporation into iTL IC1 C57BL/6NTac embryonic stem (ES) cells. Recombinant ES clones were microinjected into BALB/c blastocytes and resulting chimeras were mated with wild-type (WT) C57BL/6 mice to generate F1 heterozygotes. Correct targeting was confirmed by PCR with primers hybridizing to the neo gene (5-GTACTGTATCAGTAGACATTGG-3) and a flanking primer 3' to the short homology arm (5-CCAGAGGCCACTTGTGTAGC-3). The neomycin cassette was then removed by breeding heterozygous offspring to ACTFLPe mice (Jackson Labs, Bar Harbor, ME). Mice that no longer contained the FLPe transgene but retained the loxP sites were intercrossed to generate homozygote *Oprk1*-floxed mice (KOR$^{lox/lox}$). We then crossed KOR$^{lox/lox}$ with mice expressing Cre during early embryonic development (EIIa-Cre; Jackson Labs, Bar Harbor, ME), thereby generating constitutive KOR knockouts (KOR$^{-/-}$), and with mice expressing Cre in dopamine transporter-containing cells (DAT-Cre;

Parlato et al., 2006), thereby generating conditional knockouts that lack KORs in DA-containing neurons (DAT-$KOR^{lox/lox}$). Mice were backcrossed to C57BL/6J (7 generations) before testing. For experiments, $KOR^{-/-}$ mice and littermate controls ($KOR^{+/+}$) were obtained by breeding KOR heterozygous ($KOR^{+/-)}$ mice. DAT- $KOR^{lox/lox}$ and littermate controls ($KOR^{lox/lox}$) were obtained by breeding floxed KOR mice expressing the DAT-Cre transgene with floxed mice lacking the Cre transgene.

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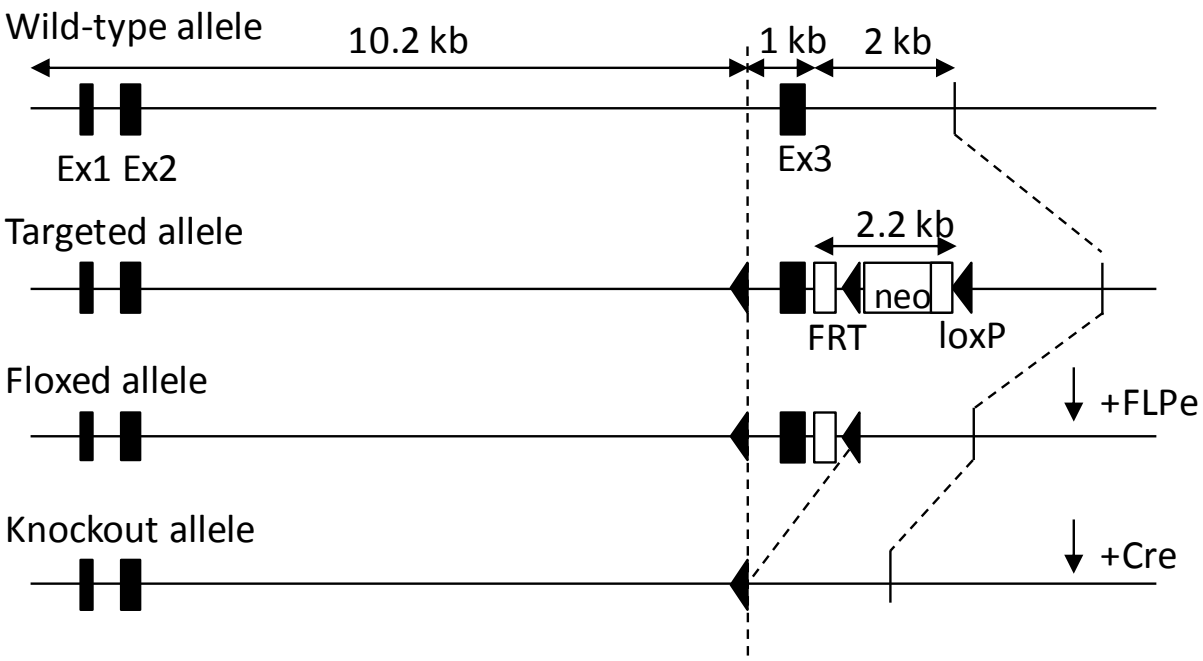


Figure 4.1 Generation of KOR-deficient mice. Representative depiction of the wild-type, targeted, floxed, and KO alleles. The neo cassette was removed from the targeted allele by FLPe-induced recombination. KO mice were generated by breeding to Ella- and DAT-Cre mouse lines to generate constitutive KOs and conditional KOs with KORs ablated in DA neurons. DA, dopamine; KO, knockout; KOR, kappa-opioid receptor

Genotyping: Genomic DNA samples obtained from tail biopsies were tested by polymerase chain reaction (PCR) to demonstrate recombination at the floxed KOR allele using primers annealing immediately upstream of each loxP site and just downstream of the floxed region (5-TATTGCTGACCTATCGTGAAC-3, 5-GAGCTACTGTTTTCATACCATTTA-3, and 5-TTGAGGCTACTAGTTTCCAAAG-3). PCR products were run on a 1% agarose gel. Expected fragment sizes were 399 base pairs (bp) for WT DNA, 459 bp for unrecombined DNA and 563 bp for KOs. PCR cycling conditions were 94°C, 45 sec; 60°C, 20 sec; 70°C, 1 min for 40 cycles. To determine whether mice expressed the Cre transgene, genotyping was performed with primers to iCre (5-GTGGATGCCACCTCTGATGAAGTCAGGA-3 and 5-CAATGCGCAGCAGGGTGTTGTAGGCAAT-3).

Autoradiography: Autoradiography was performed using [^{3}H]U69,593 (PerkinElmer, Waltham, MA), a tritiated version of a highly selective KOR agonist (La Regina et al., 1988), as described previously (Wang et al., 2011). Mice were killed by decapitation, and brains were removed and immediately immersed in isopentane on dry ice. Coronal sections (20 μm) were cut on a cryostat (Leica CM3050 S) maintained at -20°C, thaw-mounted onto gelatin-dipped slides, and stored at -80°C until processed. Sections were incubated with 5 nM [^{3}H]U69,593 in 50 mM Tris-HCl buffer (pH 7.4) for 1 hour at 25°C. Non-specific binding was assessed in the presence of 10 μM naloxone, a non-specific opiate receptor inhibitor. Slides were then rinsed three times (2 minutes each) in cold 50 mM Tris-HCl buffer, pH 7.4, and once (30 seconds) in deionized H_2O, dried under a cool stream of air, and exposed to tritium-sensitive storage phosphor screens for 3 weeks in cassettes. Radioactive images captured on phosphor screens along with

[^{3}H]microscale standards (GE Healthcare, Buckinghamshire, UK) were visualized using a Cyclone Storage Phosphor Scanner (Packard Bioscience), and data were analyzed using the OptiQuant program. Nonspecific binding was subtracted from total binding and the resultant values represent specific [^{3}H]U69,593 binding in fmol/mg as determined using [^{3}H]microscale. Data are reported as mean values plus standard error of the mean (SEM) from three to four sections in each brain.

Quantitative reverse transcription PCR (qPCR): To complement the autoradiography studies, we performed qPCR on cDNA synthesized from tissue punches of the NAc, caudate putamen (CPu), amygdala (AMY), and midbrain (comprising the VTA plus substantia nigra (VTA/SN). Frozen brains were coronally sectioned on a cryostat (-20°C) until the rostral NAc was exposed (Bregma 1.70 mm). Bilateral 1-mm^3 punches of NAc tissue were taken and kept on dry ice. More caudal brain regions of interest were sequentially obtained by sectioning until the rostral face of the region was exposed and bilateral tissue punches obtained (average tissue weight≈15.0 mg). To verify the location of the tissue punches, 30 µm coronal sections were collected at the beginning and end of each punch; these sections were slide mounted, stained with 0.1% cresyl violet, and analyzed to verify that the tissue punch location was within targeted brain regions. Total RNA was extracted using PureLink RNA Mini Kit (Invitrogen). RNA quality and quantity were assessed using an RNA 6000 Nano Chip (Agilent, Santa Clara, CA) on an Agilent Bioanalyzer 2100. RNA integrity number (RIN) exceeded 7 for all samples, indicating high quality. 500 ng of total RNA was used to synthesize cDNA using iScript cDNA Synthesis Kit (BioRad) in a ThermoHybaid iCycler (Thermo Scientific). Primers specific for KOR (*Oprk1*; 5-TCCTTGGAGGCACCAAAGTCAGGG-

3, and 5-TGGTGATGCGGCGGAGATTTCG-3), DAT (*Slc6a3*; 5-AATGCCCTGGGCTGGATCATTGC-3 and 5-AATGGCGCAGCGTGAATTGGC-3), β-actin (*Nba*; 5-AGTGTGACGTTGACATCCGTA-3 and 5-GCCAGAGCAGTAATCTCCTTCT-3), and filamin β protein (*FlnB*; 5-TTCACTGTGGGCGTTGCTGC-3 and 5-AAGCATGGCACCACCTTCCG-3) genes were designed using NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and purchased from Integrated DNA Technologies (Coralville, Iowa). The KOR primer set flanked the exon 3 to exon 4 junction. Melt curve analysis and polyacrylamide gel electrophoresis confirmed the specificity of the primers. The amplicon bp lengths are 237 (KOR), 178 (DAT), 112 (β-actin), and 176 (FlnB).

The qPCR was run using the iQ SybrGreen Supermix (BioRad). The reaction was carried out on a MyiQ Single Color Real-Time PCR Detection System (BioRad) in a volume of 20 µl, with 2.0 µL of 3.0 µM forward and reverse primers, 2.0 µL Rnase/Dnase free H_2O, 10 µL SybrGreen Supermix, and 4.0 µL cDNA sample diluted 1:10. PCR cycling conditions were 95°C for 5 min; 40 cycles at 94°C for 15 sec, 60°C for 15 sec, 72°C for 15 sec. Data were collected at a read temperature of 81°C to 83°C for 15 sec depending on the amplicon melt temperatures. Standard dilution curves were generated for each primer in every experiment and on every plate by serially diluting (1.00, 0.25, 0.0625, and 0.0156-fold) a master cDNA stock comprising an equal mix of cDNA pooled from the brain regions of interest of all control mice. The undiluted master sample was assigned an arbitrary concentration of 1.00. MyiQ Optical System Software (BioRad) was used to analyze the data, and reported values for KOR and DAT were normalized to the average of two internal controls: β-actin, and FlnB, neither of

which showed group differences in qPCR analyses. Samples containing no cDNA template and samples from cDNA reactions containing no reverse transcriptase were run as controls for contamination and amplification of genomic DNA, respectively. All samples were run in duplicate. In experiments that compared gene expression in the NAc, CPu, AMY, and VTA/SN, qPCR reactions for KOR and internal control gene analysis were run concurrently with all samples from these regions on the same 96 well plate, to ensure identical amplification and measurement conditions.

Cre-recombinase immunohistochemistry: Mice were perfused with 0.9% saline followed by 4% paraformaldehyde. Whole brains were extracted and postfixed in 4% paraformaldehyde overnight then transferred to 30% sucrose for cryoprotection. Brains were sectioned at 30 µm and incubated in 0.3% H_2O_2 for 30 min, then blocked in 5% normal goat serum (Vector Laboratories, Inc., Burlingame, CA), for 1 hour. Sections were incubated in primary antibody to Cre-recombinase (1:2,000; Millipore, Billerica, MA) in 5% NGS overnight. The next day sections were incubated in biotinylated anti-rabbit IgG (1:500; Vector Laboratories, Inc., Burlingame, CA) for 1 hour followed by ABC for 1 hour (ABC Elite Kit, Vector Laboratories, Inc., Burlingame, CA). Cre-recombinase immunoreactivity was visualized with DAB.

Warm water tail-flick assay: The tail-flick assay was used to assess KOR-mediated analgesia (Janssen et al., 1963; McLaughlin et al., 2003a). Mice were held by the scruff and their tails were submerged 1-2 cm in a 52±1°C water bath. The latency for mice to withdraw their tail from the bath was timed with a stopwatch to establish baseline

withdrawal latency. Mice were then treated with the KOR agonist (±)U50,488 (15 mg/kg IP) and withdrawal latencies were assessed 30 min later. A maximum tail emersion time of 15 sec was used to prevent tissue damage.

Acoustic startle response: Acoustic startle data were collected by measuring the amplitude of the startle reflex in response to white noise bursts using the Med Associates Inc. (St. Albans, VT) Startle Reflex System and Advanced Startle software program. Mice were placed into Plexiglas cages (8.5 x 7 x 7 cm) with steel rod floors attached to load cell platforms contained inside sound and light attenuating chambers (40 x 64 x 42 cm). The load cells are capable of transducing movement into an electrical output that is amplified and digitized into arbitrary units by an analog-to-digital converter interfaced to a personal computer. Startle amplitude was defined as the maximum peak-to-peak voltage occurring within the first 100 ms after the onset of the startle stimulus. An audio stimulator generated 40 msec startle stimuli (1-32 kHz white noise) that were delivered through speakers located behind the cages. To assess their startle threshold mice were allowed 5 min in the chamber to acclimate followed by 100 startle stimuli, 10 each at 70, 74, 78, 82, 86, 90, 94, 98, and 102 dB in a pseudo-random order with an interstimulus interval of 7-23 sec (average of 15 sec).

Weight: To determine if KOR deletion alters body weight, individual mice were weighed at weekly intervals beginning at weaning (4 weeks) and continuing until adulthood (8 weeks). The same scale was used throughout the experiment and mice were only included if their exact date of birth was known. Weights were recorded to 0.1 gm.

Visual cliff: Depth perception (visual acuity) was tested in the visual cliff paradigm. The apparatus consisted of a platform 1 meter above the ground with a checkered pattern. A clear piece of Plexiglas was placed on the platform and extended 0.5 meters beyond the platform edge. The checkered pattern was also placed on the floor below the extending Plexiglas, giving the illusion of an edge. At this interface was a smooth beam (2.54 x 2.54 cm) running the length of the edge. Mice were placed on the beam and allowed to step off to either side. Choices were recorded as safe if the mouse stepped toward the platform side and unsafe if the mouse chose the overhang side. Each mouse was tested for 10 trials.

Activity testing: To determine if KOR deletion alters overall locomotor activity, mice were tested in clear Plexiglas open-field arenas (28 x 28 x 20 cm) housed inside dark light and sound attenuating chambers (Med Associates Inc., St. Albans, VT, USA). Locomotor activity was quantified using an automated system equipped with infrared beams. Beam breaks were recorded and converted into horizontal distance travelled (Activity Monitor 5.0; Med Associates Inc.). The testing period was 1 h. The amount of time mice spent in the 16 x 16 cm interior of the open field (Center Time), a metric increased by drugs with anxiolytic effects in people (Prut and Belzung, 2003; Knoll et al., 2007), was also quantified, although only the final 45 min of these data were analyzed.

Cocaine sensitization: The cocaine locomotor sensitization test paradigm began immediately after the initial 1 hr activity test. The methods were similar to those previously described (Chefer et al., 2005). Briefly, at the same time each day mice

were subjected to a 3 hr test which included a 1 hr habituation session (0-60 min), a 1 hr post saline (10 mL/kg, intraperitoneal [IP]) test session (60-120 min), and a final 1 hr post cocaine (15 mg/kg, IP) test session (120-180 min). This procedure was repeated daily for 5 consecutive days followed by two days off and a final identical test on day 8.

Cocaine place conditioning: The unbiased place conditioning apparatus consisted of opaque Plexiglas chambers with two interchangeable and distinct tactile floor stimuli (hole vs. grid) with which cocaine or vehicle injections were paired. Each chamber included a removable guillotine door that allowed the confinement of mice to one half of the apparatus. The experiments proceeded in three phases: habituation (one session), conditioning (4 sessions; 2 CS+ and 2 CS-), and preference testing (one session). The habituation session was intended to reduce the novelty of the experimental apparatus and injection procedures. On the first day of the experiment, mice were injected with vehicle just prior to being placed in the conditioning chamber for 30 min, where the floor was lined with smooth Plexiglas and the entire apparatus was open for exploration. During the conditioning phase, mice were injected with vehicle (AM) or cocaine (PM; 1.25 or 5 mg/kg) just before being confined to one side of the conditioning chamber for 30 min. After two conditioning trials (2 saline, 2 cocaine; Days 2–3), a 30-min preference test was conducted (Day 4) wherein the test floor was half hole and half grid and the entire apparatus was open for exploration. All conditioning and testing was conducted under dim light and scored using an automated system (Noldus EthoVision XT). Percent time in the drug-paired side was calculated based on the automated output. The apparatus was cleaned with 70% isopropyl alcohol between mice.

Light/dark box test: The light/dark box test was used as a secondary method of quantifying anxiolytic-like effects only when data from the open field suggested group differences in Center Time. New cohorts of mice were used. The apparatus consisted of a box (42 x 42 x 30 cm), one third of which was the dark compartment (2 lux) and two thirds of which was the light compartment (380 lux). An opening (10x5 cm) allowed passage between the two sides. Mice were placed into the dark compartment and allowed to move freely in the apparatus for 5 min. The latency for mice to transition from the dark chamber to the light chamber and the total time in the light chamber was recorded with EthoVision XT (Noldus Information Technology, Wageningen, Netherlands). The apparatus was cleaned with 70% isopropyl alcohol between mice.

Elevated plus maze: Mice that were tested in the light/dark box were also tested 7 days later in the elevated plus maze. Mice were tested on a plus maze (75 x 75 cm) that was elevated 1 m above the floor. Each arm was 35 x 6 cm connected by a 5 x 5 cm center area. Mice were transferred to the dimly lit testing room at least 1 hr prior to testing. Mice were placed onto the center of the apparatus facing a closed arm and activity was recorded for 5 min using EthoVision XT. Total time spent in the open arms and total distance traveled (cm) were quantified. The maze was cleaned with 70% isopropyl alcohol between mice.

Data analysis: All data are graphed as mean plus SEM. Data for each genotype were analyzed by two-sample t-tests or ANOVA followed by Bonferroni *post hoc* tests as appropriate. For the visual cliff and place conditioning experiments, one sample t-tests were used to compare the observed data for each genotype to the hypothetical data

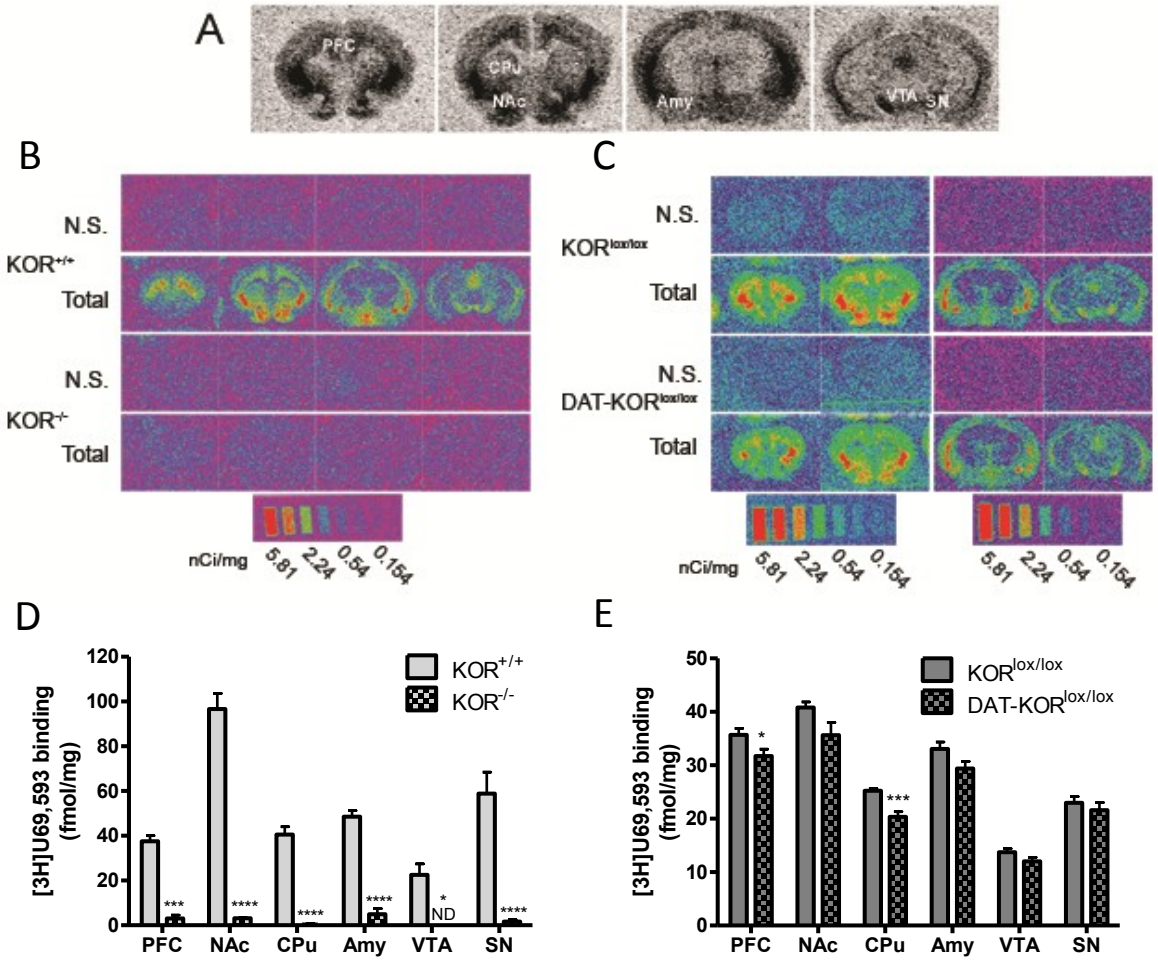
expected for blind mice or lack of cocaine preference respectively. Startle threshold was calculated as the lowest dB significantly different from no stimulation response using separate ANOVAs for each genotype.

Results

Radioligand binding of [^{3}H]U69,593 was used to determine whether the floxed construct was effective in eliminating functional KORs in KOR-mutant mice. No detectable [^{3}H]U69,593 binding was present in KOR$^{-/-}$ mice, whereas autoradiographic mapping in WT littermates showed clear distribution of KORs throughout the brain including the NAc, AMY, and cortex (**Fig. 4.2B**). The distribution is similar to those reported previously (reviewed in Mansour et al., 1988). Quantification demonstrated a significant difference in [^{3}H]U69,593 binding in brain areas of the mesocorticolimbic DA system (PFC, NAc, caudate-putamen, AMY, VTA and SN), depicted in **Fig 4.2A**. Binding in KOR$^{-/-}$ mice was reduced to background levels in all regions analyzed (*P's*<0.05; **Fig. 4.2D**). [^{3}H]U69,593 binding was visibly similar in both DAT-KOR$^{lox/lox}$ mice and littermates (KOR$^{lox/lox}$) (**Fig. 4.2C**), but quantification showed that DAT-KOR$^{lox/lox}$ mice had reduced binding in the PFC and CPu (*P's*<0.05; **Fig. 4.2E**). Since KORs are also expressed in non-dopaminergic cells, the reductions in any particular region of conditional KOs may represent only a fraction of total KORs in that area.

Figure 4.2 [^{3}H]U69,593 binding in KOR KO lines. ***A.*** Representative autoradiograms of [^{3}H]U69,593 binding to KORs in coronal brain sections of a KOR$^{+/+}$ mouse at 4 different levels from rostral to caudal positions depicting regions of interest quantified in ***D,E*** . ***B,C.*** Representative computer-generated pseudocolor autoradiograms of [^{3}H]U69,593 binding in KOR$^{+/+}$, KOR$^{-/-}$, KOR$^{lox/lox}$, and DAT-KOR$^{lox/lox}$ mice. Non-specific binding (N.S.) was assessed in the presence of naloxone. Note that exposure of sections to different screens yields slightly different signals, which were then calibrated with the corresponding microscale standards. ***D,E.*** Quantification of specific [^{3}H]U69,593 binding (fmol/mg tissue) from brain regions known to express KORs. **P*<0.05, ****P*<0.001, *****P*<0.0001 (n=3-6). AMY, amygdala; CPu, caudate-putamen; NAc, nucleus accumbens; PFC, prefrontal cortex; SN, substantia nigra; VTA, ventral tegmental area

Figure 4.2 (Continued) [^{3}H]U69,593 binding in KOR KO lines



To further confirm the specificity of the deletion, brain dissections (punches) were analyzed by qPCR to quantify mRNA levels. We verified this technique by examining KOR and DAT mRNA in $KOR^{-/-}$ mice (gene x genotype interaction, $F[2,9]=39.94$, $P<0.0001$). *Post hoc* analysis revealed reduced levels of KOR mRNA in heterozygotes and no detectable mRNA in $KOR^{-/-}$ mice compared to $KOR^{+/+}$ controls (**Fig. 4.3A**). The accuracy of the brain punches was verified through analysis of DAT mRNA, which showed similar levels among the genotypes. KOR mRNA levels were reduced in the VTA/SN of DAT-$KOR^{lox/lox}$ mice compared to littermate controls whereas DAT mRNA levels were not significantly different (gene x genotype interaction, $F[1,6]=20.35$, $P<0.01$; **Fig. 4.3B**). As expected based on the location of Cre expression (**Fig. 4.4**), this reduction was specific to the VTA/SN and was not observed in other brain regions (region x genotype interaction, $F[3,18]=8.10$, $P<0.01$; **Fig. 4.3C**).

Since KOR activation induces analgesia, the tail flick assay was used as a behavioral confirmation of KOR deletion. Baseline tail withdrawal latencies did not differ between $KOR^{-/-}$ or DAT-$KOR^{lox/lox}$ mice and their littermate controls (**Fig. 4.5A**). In $KOR^{-/-}$ mice, tail withdrawal latencies depended upon a drug x genotype interaction ($F[1,31]=24.23$, $P<0.0001$; **Fig. 4.5A**). After U50,488 treatment, $KOR^{+/+}$ mice had increased tail withdrawal latency compared to their own baseline ($P<0.0001$) and significantly longer latencies than $KOR^{-/-}$ mice ($P<0.0001$). The response to U50,488 was similar to that observed in WT mice (increased latency compared to baseline) in DAT-$KOR^{lox/lox}$ mice and did not differ compared to littermate controls (main effect of drug, $F[1,21]=24.92$, $P<0.0001$; **Fig. 4.5B**), suggesting that DAT-expressing cells make a minimal contribution to the antinociceptive effects of KOR agonists. While some differences in

the baseline and drug responses were noted between the mutant strains, this likely represents variability between the individual experiments, since all mice within each experiment were tested on the same day.

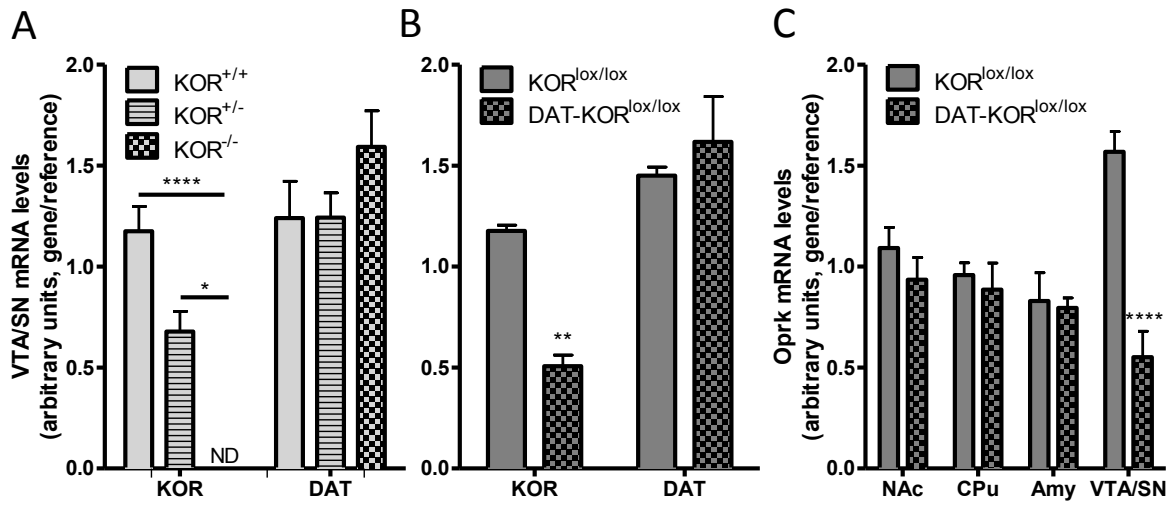


Figure 4.3 Gene expression analysis using qPCR in KOR mutant mice. ***A.*** Levels of KOR mRNA from punches of the ventral tegmental area and substantia nigra (VTA/SN) of mutant mice indicates reduced KOR mRNA in $KOR^{-/+}$ mice and no detectable levels in $KOR^{-/-}$ mice compared to controls. ***B.*** Similarly, DAT-$KOR^{lox/lox}$ mice have significantly less KOR mRNA than $KOR^{lox/lox}$ littermates. Levels of DAT mRNA were equivalent in mutants and controls. ***C.*** KOR mRNA reductions were specific for the VTA/SN in DAT-$KOR^{lox/lox}$ knockouts. **P*<0.05, ***P*<0.01, *****P*<0.0001 (n=4). qPCR, quantitative reverse transcription polymerase chain reaction

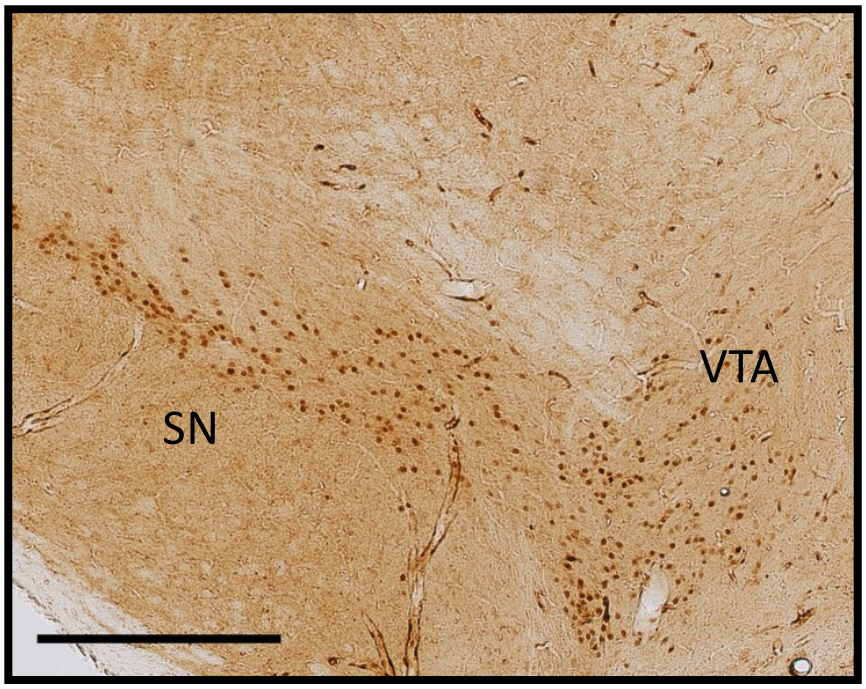


Figure 4.4 Immunohistochemistry for Cre-recombinase. DAT-KOR$^{lox/lox}$ mice show Cre-recombinase immunoreactivity specifically in the VTA and SN. Scale bar indicates 400 μm.

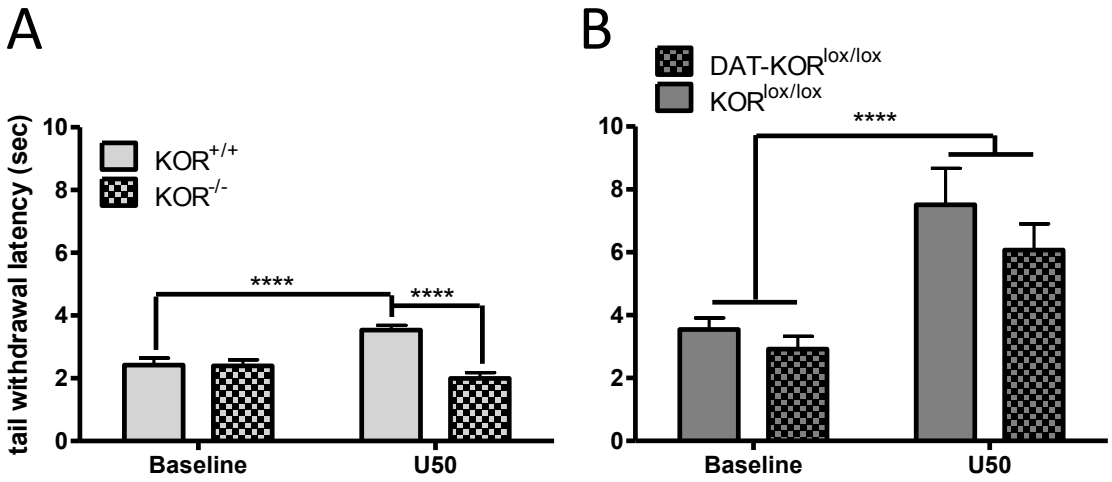


Figure 4.5 Warm water tail-flick assay. Mice were tested at baseline and 30 min following the KOR agonist U50,488 (15 mg/kg, IP). ***A.*** U50,488 induced analgesia in $KOR^{+/+}$ but not $KOR^{-/-}$ mice. ***B.*** U50,488 significantly increased tail withdrawal latencies in both $KOR^{lox/lox}$ and DAT-$KOR^{lox/lox}$ knockouts. ****P<0.0001 (n=11-17).

Both strains of the mutant mice appeared normal upon gross examination. Regardless, a series of experiments were conducted to determine whether their basic development and senses were intact. Mice were weighed weekly from age 4 to 8 weeks (**Fig. 4.6A,D**) and analysis of mean body weight by time and genotype revealed a main effect of weight over time ($F[4,84]=388.40$, $P<0.0001$; $F[4,64]=535.70$, $P<0.0001$); however, both mutants were virtually indistinguishable from their littermate controls. To determine if hearing was affected in KOR-deficient mice, startle threshold was determined in both lines (**Fig. 4.6B,E**). Startle amplitude increased with increasing sound intensity (dB) but did not differ significantly between control and mutant mice in either $KOR^{-/-}$ or DAT-$KOR^{lox/lox}$ mice. To calculate startle threshold, each genotype was analyzed separately. The lowest sound intensity tested that was significantly different from no stimulation controls was 86 dB in all groups indicating similar thresholds in control and mutant mice. Vision was tested in the visual cliff paradigm (**Fig. 4.6C,F**). The percent of safe choices over 10 trials was determined for each mouse. Mutant mice did not differ from controls, or show deficits in visual depth perception as indicated by percent safe choices significantly above chance (50%; *P*'s<0.01). This is consistent with other studies in sighted mice, and in contrast to the random (52% safe) choices made by a blind strain of mice (C3H/HeJ; Fox, 1965). Overall, these data indicate that neither constitutive nor DA neuron-selective KOR deletion has an effect on body weight or basic sensory functions such as hearing or vision.

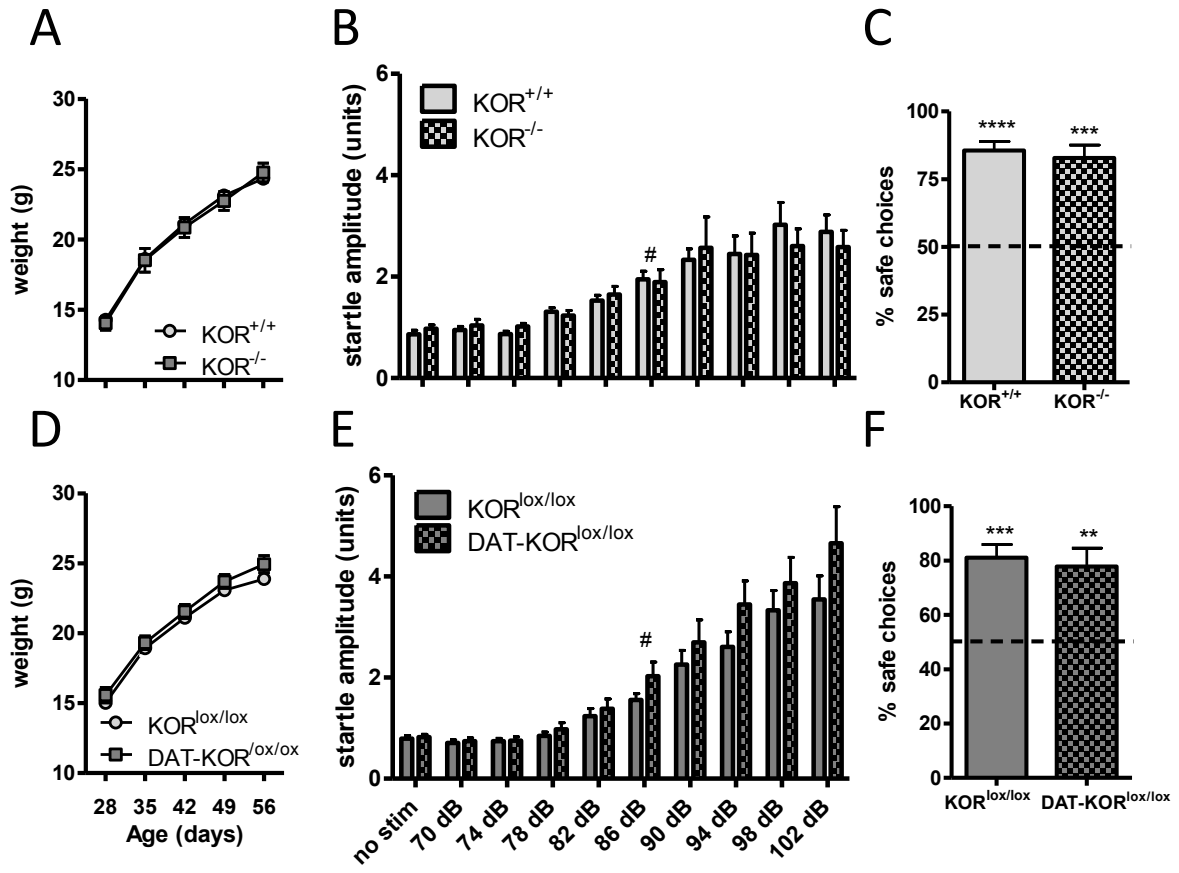


Figure 4.6 Effects of KOR deletion on weight gain and sensory function. ***A,D.*** Weight gain in KO mice did not differ from littermate controls. (n=9-14) ***B,E.*** Startle responding in KO mice did not statistically differ from controls. Hearing thresholds for all genotypes was 86 dB. (n=8-10) ***C,F.*** Vision was not affected in either KOR KO line. (n=7-9) #*P*<0.05 compared to no stimulation (no stim) condition. Each genotype was analyzed individually. ***P*<0.01, ****P*<0.001, *****P*<0.0001 compared to 50%.

Activity testing also did not reveal any differences between mutant mice and controls (**Fig. 4.7A,C**). A more detailed analysis of the activity data suggested some strain differences in time spent in the center of the open field. Although there were no significant differences in Center Time when the entire 60-min test period was analyzed (not shown), visual inspection of the data indicated high variability during the first 15 min—a time which mice were most actively exploring the new environment—followed by a 45-min period during which group differences emerged. As such, we re-analyzed the Center Time data using only the final 45 min of the test session. Under these conditions, $KOR^{-/-}$ mice were similar to WT controls in Center Time (main effect of time, $F[2,28]=6.48$, $P<0.01$; **Fig. 4.7B**). However, DAT-$KOR^{lox/lox}$ mice spent considerably more time in the center of the open field (main effect of genotype, $F[1,32]=5.45$, $P<0.05$; **Fig. 4.7D**), an anxiolytic-like effect. To determine whether these mice would show less anxiety-like behavior in other paradigms, DAT-$KOR^{lox/lox}$ mice were tested in the light/dark box and elevated plus maze. DAT-$KOR^{lox/lox}$ mice had significantly shorter latencies to enter the light side of the light/dark box ($P<0.05$, anxiolytic-like effect; **Fig. 4.8A**), although once making this transition the total time in the light side did not differ between genotypes (**Fig. 4.8B**). Open field and light/dark box effects were not attributable to the expression of Cre recombinase alone, since DAT-$KOR^{+/+}$ mice did not differ from $KOR^{+/+}$ littermate controls (**Fig. 4.7D,E; Fig. 4.8C,D**). No differences were detected in open arm time or open arm entries in the elevated plus maze (**Fig. 4.8E-H**).

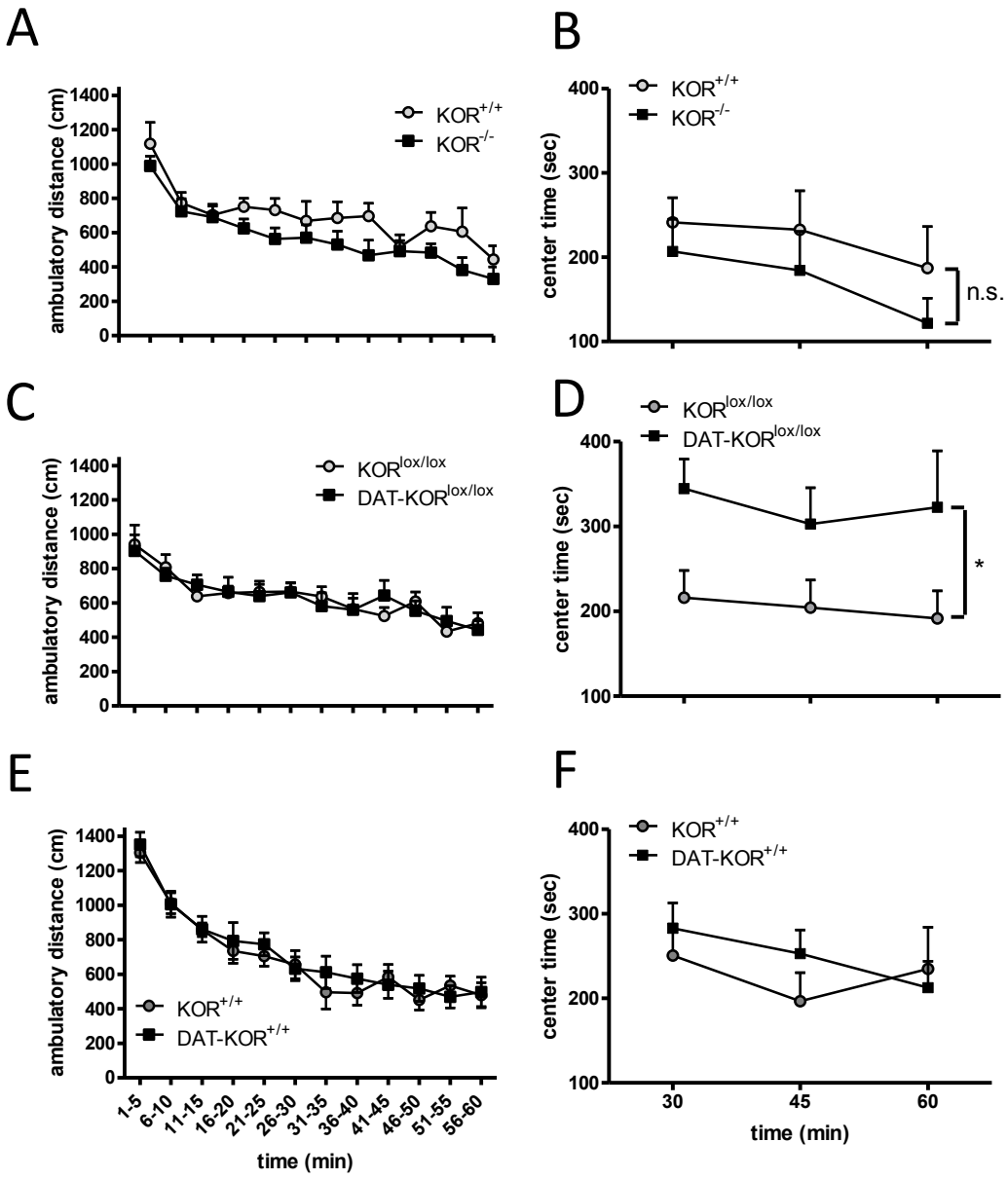
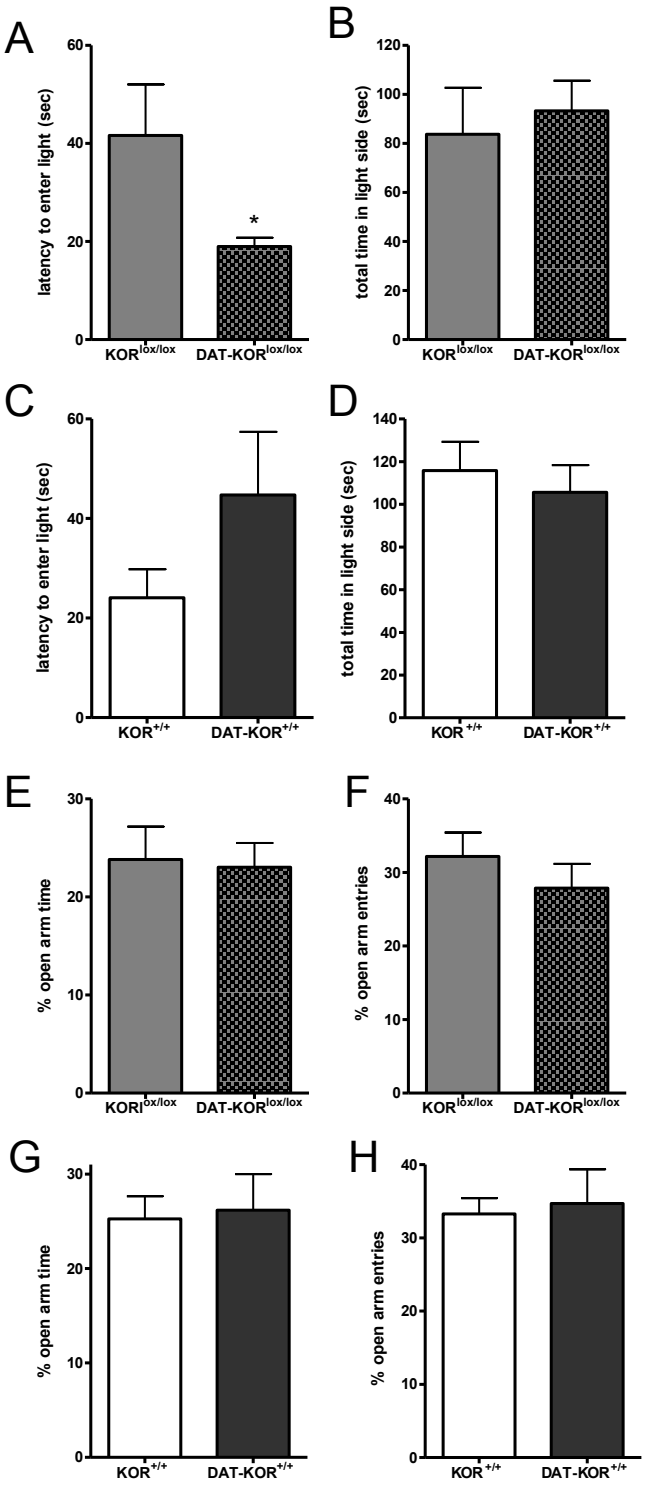


Figure 4.7 Open field activity. ***A,C.*** General locomotor behavior was unchanged by KOR deficiency. ***B.*** Time spent in the center of the open field was not significantly different between $KOR^{+/+}$ and $KOR^{-/-}$ mice. ***D.*** DAT-$KOR^{lox/lox}$ mice spent significantly more time in the center than $KOR^{lox/lox}$ littermates. **P*<0.05 (n=8-9) n.s., not significant

Figure 4.8 Effects of KOR ablation in DA neurons on light/dark box and EPM behavior. ***A.*** DAT-KOR$^{lox/lox}$ mice had reduced latencies to first enter the light compartment compared to KOR$^{lox/lox}$ controls. ***B.*** No differences were observed in total time spent in the light compartment. ***C,D.*** In the EPM, DAT-KOR$^{lox/lox}$ mice did not differ from KOR$^{lox/lox}$ controls in the percent time spent in the open arms or percent open arm entries. *P<0.05 (n=10-11). EPM, elevated plus maze

Figure 4.8 (Continued) Effects of KOR ablation in dopamine neurons on light/dark box and elevated plus maze behavior



Previous results suggest that $KOR^{-/-}$ mice fail to exhibit cocaine-induced behavioral sensitization, but appear pre-sensitized to the locomotor activating effects following the first injection of cocaine (Chefer et al., 2005). To determine whether our lines of mice—which differ from these previous lines because they were generated by Cre-loxP recombination—exhibit a similar behavioral phenotype, mice were tested in a cocaine-induced behavioral sensitization paradigm. Both $KOR^{-/-}$ mice and $KOR^{+/+}$ littermates developed locomotor sensitization to cocaine and did not significantly differ from one another (**Fig. 4.9A-C**). Analysis of distance travelled in the 1 hour following cocaine treatment revealed a main effect of day ($F[5,70]=12.11$, $P<0.0001$; **Fig. 4.9C**), but no interaction with genotype. Since behavioral sensitization may involve changes in DA function (Koff et al., 1994; Valjent et al., 2010), mutant mice lacking KORs specifically in DAT-expressing neurons were also tested. Both DAT-$KOR^{lox/lox}$ mice and littermate controls exhibited locomotor sensitization to daily injections of cocaine, although the degree of sensitization was enhanced in the mutants**,** and depended upon a genotype x treatment day interaction ($F[5,80]=2.64$, $P<0.05$; **Fig. 4.10A-C**). Post hoc analysis at each day of testing indicated that DAT-$KOR^{lox/lox}$ mice had a significantly greater response to cocaine at day 8 compared to littermate controls ($P<0.05$; **Fig. 4.10C**). Control mice expressing DAT-Cre with WT KOR alleles (i.e., lacking loxP sites; DAT-$KOR^{+/+}$) and their littermate controls ($KOR^{+/+}$) were tested separately. Cre expression alone was not sufficient to increase sensitization (data not shown).

Figure 4.9 Cocaine-induced locomotor sensitization in KOR$^{-/-}$ mice. KOR$^{-/-}$ mice were not significantly different from KOR$^{+/+}$ littermates. ***A,B.*** Time course of ambulatory distance (cm) in KOR$^{+/+}$ and KOR$^{-/-}$ mice during the first 1 hour of testing and following injection of saline (10 mL/kg) and cocaine (15 mg/kg). Time of injection is indicated by a dotted line. ***C.*** Sum of the distance travelled during the 1 hour test period following cocaine injection for each test day (n=8).

Figure 4.9 (Continued) Cocaine-induced locomotor sensitization in KOR$^{-/-}$ mice

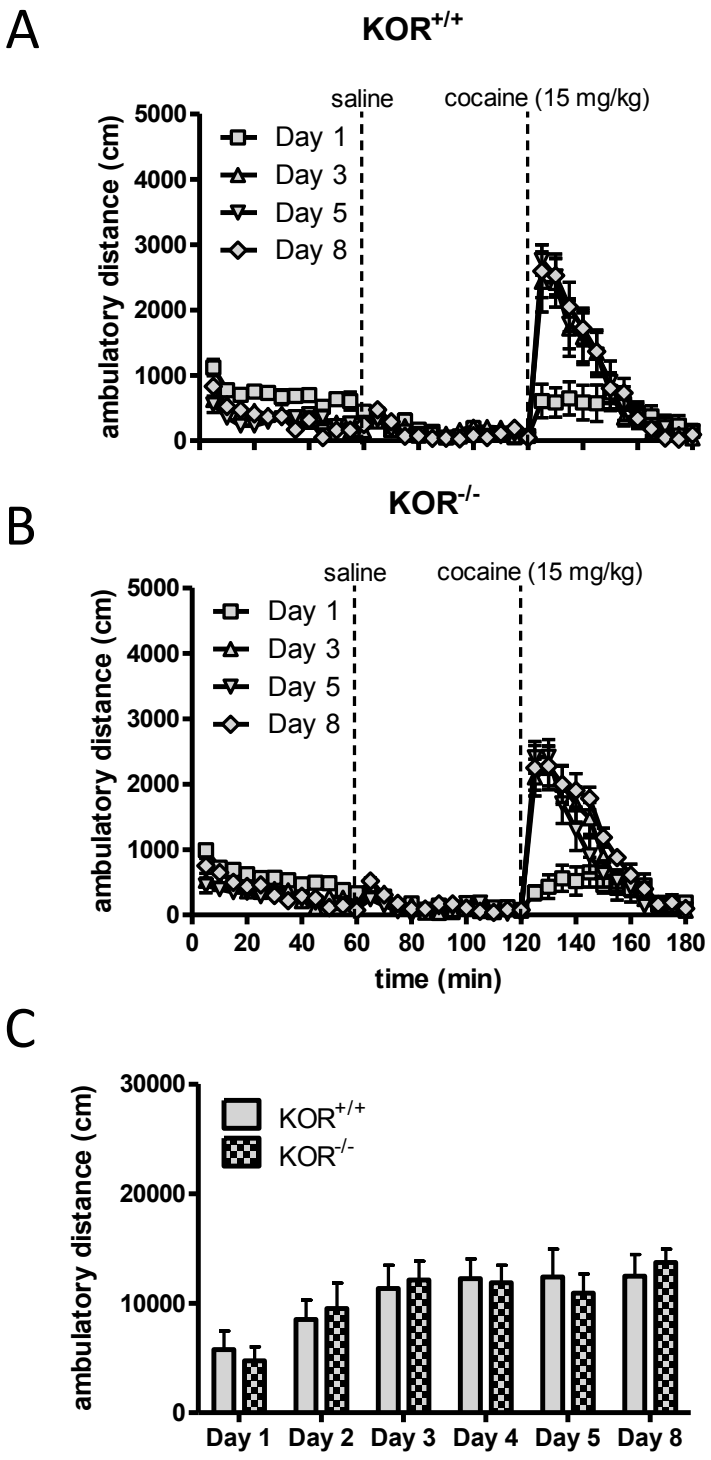
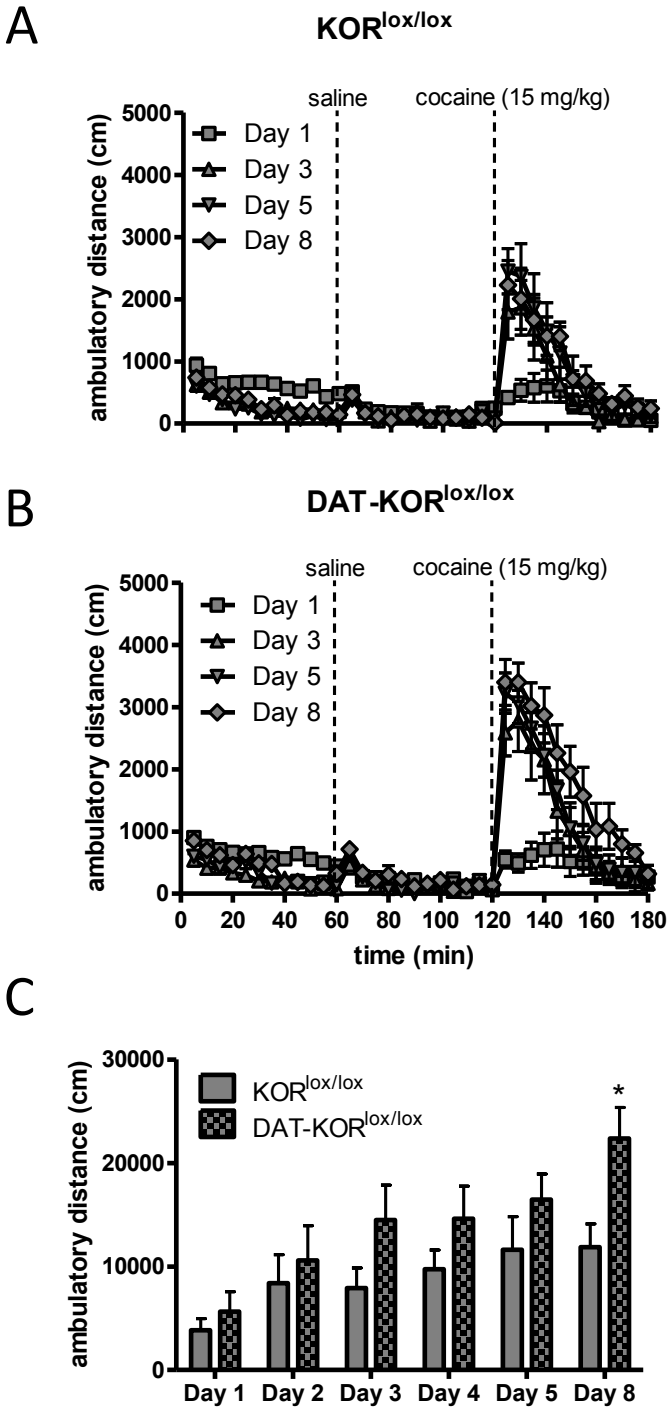


Figure 4.10 Cocaine-induced locomotor sensitization in DAT-KOR$^{lox/lox}$ mice. DAT-KOR$^{lox/lox}$ mice had greater locomotor sensitization to cocaine than KOR$^{lox/lox}$ littermate controls. ***A,B.*** Time course of ambulatory distance (cm) in KOR$^{lox/lox}$ and DAT-KOR$^{lox/lox}$ mice during the first 1 hour of testing and following injection of saline (10 mL/kg) and cocaine (15 mg/kg). Time of injection is indicated by a dotted line. ***C.*** Sum of the distance travelled during the 1 hour test period following cocaine injection for each test day. *P<0.05 (n=9).

Figure 4.10 (Continued) Cocaine-induced locomotor sensitization in DAT-KOR$^{lox/lox}$ mice



To determine whether the DAT-KOR$^{lox/lox}$ mice would also exhibit enhanced effects in other cocaine-induced behaviors, mutant mice were tested in a cocaine conditioned place preference paradigm. Mice were first tested with two conditioning trials of 5 mg/kg cocaine, the minimum dose we have found to be effective at producing place preference in mice (A.J. Bechtholt and A. Van't Veer, unpublished observations). At this dose, both genotypes displayed a significant preference for the drug paired side in contrast to the expected observation of equal time spent on both sides of the apparatus if no preference was formed (*P*'s<0.01; **Fig. 11A**) and were not different from each other. To determine if a lack of difference in genotypes was due to a ceiling effect on preference, a lower dose of cocaine (1.25 mg/kg) was used in a follow-up experiment. It was predicted that if DAT-KOR$^{lox/lox}$ mice were more sensitive to the effects of cocaine, the drug might establish a place preference at a dose too low to induce preference in WT mice. However, 1.25 mg/kg cocaine did not establish place preferences in either genotype, as indicated by virtually equal amounts of time spent in both sides of the testing apparatus (**Fig. 11B**).

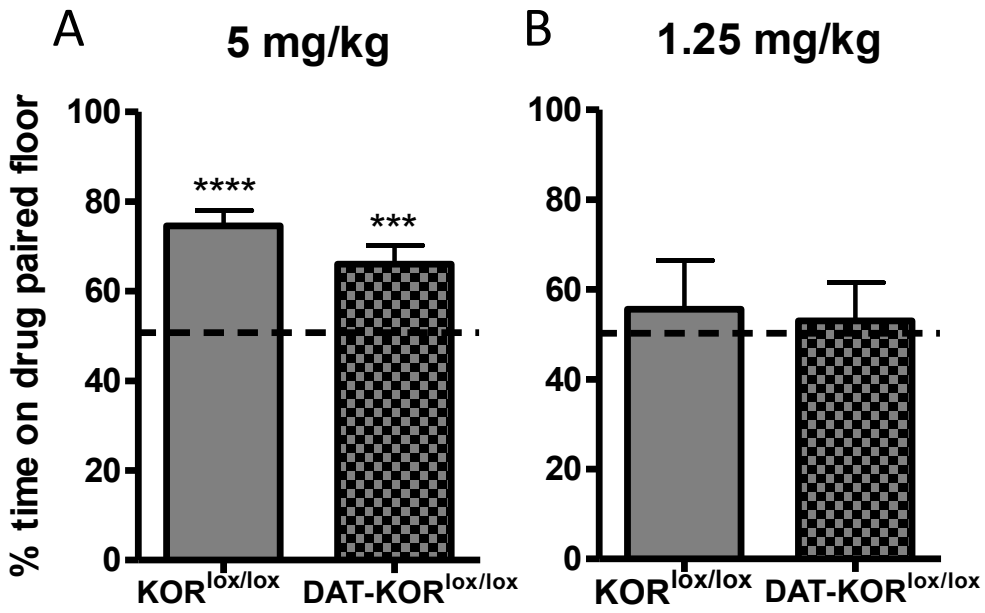


Figure 4.11 Cocaine-induced conditioned place preference. ***A.*** Place preferences observed in KOR$^{lox/lox}$ and DAT-KOR$^{lox/lox}$ mice did not differ following conditioning with 5 mg/kg cocaine. ***B.*** Neither genotype developed place preferences to 1.25 mg/kg cocaine. ****P*<0.001, *****P*<0.0001 compared to no preference (50% time on drug paired floor) (n=9-10).

Discussion

Here we describe the development of two lines of mice with mutations in KOR systems: a constitutive line ($KOR^{-/-}$) in which KORs are ablated throughout the brain and body, and a conditional line (DAT-$KOR^{lox/lox}$) in which KORs are lacking in DA-expressing cells. Autoradiography studies confirmed loss of functional KOR protein, as reflected by binding of the highly selective KOR agonist [^{3}H]U69,593. Binding of this ligand was completely absent in the $KOR^{-/-}$ mice and, as expected, reduced in the DAT-$KOR^{lox/lox}$ mice in brain areas rich in DA cell bodies or terminals while appearing intact in other brain areas. To complement these protein analyses, we used qPCR to confirm loss of KOR gene expression throughout the brain in the $KOR^{-/-}$mice and in midbrain DA systems of DAT-$KOR^{lox/lox}$ mice. In an *in vivo* test of KOR function, we found that the KOR agonist U50,488 was completely devoid of antinociceptive effects (McLaughlin et al., 2003) in the $KOR^{-/-}$ mice. However, there was no such loss of function in the DAT-$KOR^{lox/lox}$ mice, suggesting that the antinociceptive effects of KOR agonists are not mediated by brain DA systems. We also tested these mouse lines in a broad battery of tests to quantify effects of these mutations on sensory function, growth, and locomotor activity. Compared to littermate controls, both lines of mice gained weight normally throughout development and neither line of mice showed any evidence of deficits in hearing, vision, or movement in an open field (e.g., lethargy or gross motor abnormalities). Our findings suggest that the mutations we induced do not produce non-specific effects that can complicate data interpretation in tests where it is assumed that the mutants are normal in each of these domains.

While there were no differences in either strain in overall locomotor activity in the open field, a more detailed analysis in which activity patterns were quantified revealed some differences in the time spent in the center of the field (Center Time). These differences were seen only in DAT-KOR$^{lox/lox}$ mice; the KOR$^{-/-}$ mice did not differ from controls. Furthermore, Center Time differences were only statistically significant when data from the final 45 min of the test session were analyzed. The strategy of restricting data analysis to later portions of a test period is common in these types of screening procedures, especially in tests such as the forced swim test (Porsolt et al., 1977b) where there is large variability or almost complete overlap among groups in the earliest portions of the test session. Regardless, DAT-KOR$^{lox/lox}$ mice spent considerably more time in the center of the open field. This type of pattern is often interpreted as reflecting an anxiolytic-like phenotype, since drugs with anxiolytic effects in humans produce this same effect on Center Time behavior (Prut and Belzung, 2003; Knoll et al., 2007). Elevated Center Time in the KOR mutants is also broadly consistent with previous work indicating that KOR antagonists have anxiolytic-like effects in rodents (Knoll et al., 2007; Carr and Lucki, 2010; Rogala et al., 2012). To further assess the possibility that increased Center Time in the DAT-KOR$^{lox/lox}$ mice reflects an anxiolytic-like phenotype, we ran additional (naïve) cohorts of mice in the light/dark box test. Latencies to enter the light (i.e., anxiety-provoking) side of the apparatus were significantly lower in the DAT-KOR$^{lox/lox}$ mice, also suggesting an anxiolytic phenotype. However, upon entry, the mutant mice were just as likely to spend time in the light side as controls, suggesting reductions in passive avoidance behavior (latency to enter the light) rather than active avoidance behavior (tendency to escape from the light). The lack of an anxiolytic-like phenotype in the EPM is surprising, but this outcome may reflect the fact that these

mice had been used previously in the light/dark box test, which may cause generalized reductions in anxiety-related behavior in subsequent tests. Lack of effects in the EPM may also reflect the fact that this test was developed to identify pharmacological treatments (e.g., benzodiazepines) with anxiolytic effects (File, 1990; Haller et al., 2012), and may have a different threshold for identifying the more subtle effects of mutations. Lack of any evidence of an anxiolytic-like phenotype in the $KOR^{-/-}$ mutants suggests more thorough compensatory adaptations occur after constitutive receptor ablation, or that under normal conditions activation of KORs in populations of non-DA neurons produce anxiolytic effects that offset the anxiogenic effects of KOR activation in DA-expressing neurons.

Whereas the $KOR^{-/-}$ mice appeared to have normal sensitivity to the locomotor-stimulating effects of cocaine, the DAT-$KOR^{lox/lox}$ mice had exaggerated sensitivity. This exaggerated sensitivity was most evident when cocaine had been given in a repeated, intermittent (daily) regimen, followed by a 2-day period of no treatment: a challenge dose of cocaine produced much greater forward locomotion in the DAT-$KOR^{lox/lox}$ mice than in controls. The process of sensitization to the locomotor-activating effects of cocaine is often considered to be important because it is an example of cocaine-induced plasticity, and the neuroadaptations underlying this process may be related to those that contribute to addictive behaviors (Robinson and Berridge, 2000; Carlezon and Nestler, 2002). Considering that the locomotor-stimulating effects of drugs of abuse are strongly associated with their ability to elevate extracellular concentrations of DA in the NAc (Wise and Bozarth, 1987), these findings are consistent with the hypothesis that KORs provide negative regulation of the function of the

mesocorticolimbic DA system (Carlezon et al., 2009). In this context it is surprising that the $KOR^{-/-}$ mice did not show exaggerated sensitization to cocaine, considering the fact that they also lack KOR regulation of mesocorticolimbic DA systems. In addition, it has been previously reported that a different line of $KOR^{-/-}$ mice show exaggerated sensitization to the locomotor-stimulating effects of cocaine in a virtually identical treatment regimen (Chefer et al., 2005). While the line of mice used in those previous studies and our $KOR^{-/-}$ mice both have constitutive receptor ablation, there are important differences, including the targeting vector and background flanking genes (Chefer et al., 2005). Because the rewarding effects also undergo a process of sensitization with repeated exposure (Lett, 1989; Piazza et al., 1989; Shippenberg and Heidbreder, 1995); we ran additional (naïve) cohorts of the DAT-$KOR^{lox/lox}$ mice in the cocaine place conditioning test. These mice did not seem more sensitive to either a subthreshold dose of cocaine (1.25 mg/kg, IP) that does not establish a conditioned place preference in controls, or to a higher dose (5.0 mg/kg) that does establish a place preference in controls. Although these data may indicate that KORs have a more profound regulatory influence on the locomotor-stimulating effects of cocaine than on its rewarding effects, this interpretation seems unlikely considering that KOR agonists are known to reduce the reward-related effects of cocaine (Tomasiewicz et al., 2008). Rather, this effect may be due to a relative lack of sensitivity of the place conditioning test to subtle differences in reward—indeed, a common criticism of place conditioning is that dose-effects are “all or none” (Bevins, 2005)—or that more drug exposure and/or a period of no treatment is needed to reveal differential sensitivity, as was the case in the locomotor sensitization studies. Future studies that utilize other testing procedures, such as the intracranial self-stimulation (ICSS) test, may provide more insight on mutation-induced differences

in sensitivity to cocaine and other drugs of abuse. Although the ICSS procedure is very time-consuming, it is minimally sensitive to learning deficits (Carlezon and Chartoff, 2007) and it has effectively identified mutation-related alterations in sensitivity to the reward-related effects of cocaine (Roybal et al., 2007; Dinieri et al., 2009; Muschamp et al., 2012).

These mouse lines may be useful in future studies of KOR function. As one example, these lines of mice could be bred with lines expressing ligand (e.g., tamoxifen)-dependent Cre, such that the tissue selective gene excision can be induced at any time during development (Feil et al., 2009). Use of these mice would provide deeper insight on the development of long-term compensatory mechanisms that affect the phenotypes of the mutants. Similarly, viral vectors expressing Cre could be micro-infused into discrete brain areas (Berton et al., 2006; Graham et al., 2009), enabling an alternative method with which to accomplish time- and tissue-selective gene ablation. Viral vectors expressing KORs could also be used in the $KOR^{-/-}$ mice to "rescue" behavioral phenotypes or re-establish sensitivity to drugs that act at KORs. Such studies are beyond the scope of the present report, the goals of which were to characterize these mouse lines and to report the development of a novel *in vivo* tool that should be useful for studies modeling the etiology and treatment of psychiatric conditions ranging from depression and anxiety (Carlezon et al., 2009; Knoll and Carlezon, 2010) to addiction to substances including cocaine (McLaughlin et al., 2003a; Beardsley et al., 2005; Bruchas et al., 2010; Wee and Koob, 2010), nicotine (Jackson et al., 2010), and ethanol (Walker et al., 2012).

Chapter 5

Ablation of kappa-opioid receptors from dopamine neurons reduces footshock effects on startle

Ashlee Van't Veer[1], F. Ivy Carroll[2], William A. Carlezon, Jr.[1]

[1]Department of Psychiatry, Harvard Medical School, McLean Hospital, Belmont, MA 02478; [2]Research Triangle Institute, Research Triangle Park, NC

Author contributions: AVV and WAC designed the experiments. AVV conducted the experiments and analyzed the data. FIC synthesized and provided JDTic.

Abstract

Although there is strong evidence that activation of KORs expressed on midbrain DA neurons can produce depressive-like effects including anhedonia and dysphoria, their role in anxiety-like effects such as elevated startle (hyperarousal) is not known. We have shown that systemic KOR antagonism produces anxiolytic-like effects in tests of conditioned and unconditioned fear. Considering the high comorbidity of depressive and anxiety disorders, the present studies were designed to characterize the role of the mesocorticolimbic KOR system in stress-induced anxiety-like behavior. We examined startle reactivity in constitutive KOR KO mice ($KOR^{-/-}$) and conditional KOs in which KORs are selectively deleted in DA-containing neurons (DAT-$KOR^{lox/lox}$) following CRF and footshock stressors, both of which potentiate startle in WT mice. $KOR^{-/-}$ mice had similar levels of CRF-enhanced and footshock-potentiated startle compared to littermate controls. In contrast, KOR ablation restricted to DA neurons was sufficient to reduce footshock-potentiated startle, although it had no effect on CRF-enhanced startle. To complement studies in DAT-$KOR^{lox/lox}$ mice, we ablated KORs specifically in the VTA of adult $KOR^{lox/lox}$ mice by infusing a viral vector expressing Cre directly into this region. Surprisingly, startle behaviors in viral vector-treated mice did not differ from that of controls. These data provide support for a role of KORs on midbrain DA cells in the manifestation of stress, as well as some evidence that disruption of KOR function reduces stress-induced anxiety-like behavior, but they raise questions about why the various approaches to KOR ablation yield different results.

Introduction

Although stress is a normal response to adverse events, chronic or severe stress is implicated in the development of pathological anxiety, depression, and substance abuse (Kessler, 1997; Kendler et al., 1999; Volkow and Li, 2004). Stress induces the release of dynorphin (Chavkin et al., 1982; Przewlocki et al., 1987; McLaughlin et al., 2003a), and subsequent activation of KORs, which mediate its effects on behavior (Land et al., 2008; Bruchas et al., 2009). Thus KOR agonists mimic stress effects (McLaughlin et al., 2006a; Schindler et al., 2010; Bruchas et al., 2011) and produce depressive-like behaviors (Mague et al., 2003; Todtenkopf et al., 2004; Carlezon et al., 2006). Conversely, KOR antagonists reduce effects of stress (Takahashi et al., 1990; Menendez et al., 1993a; McLaughlin et al., 2003a; McLaughlin et al., 2006b; Carey et al., 2009; Sperling et al., 2010) and are antidepressant- and anxiolytic-like (Mague et al., 2003; Beardsley et al., 2005; Knoll et al., 2007; Carr et al., 2010). Together, these data support the hypothesis that KOR activation plays an important role in the etiology of stress-induced anxiety and depressive disorders.

KORs are expressed throughout the brain in humans and rodents, particularly in regions associated with anxiety and depressive behavior such as the VTA, NAc, AMY, HIP, and BNST (Fallon and Leslie, 1986; Mansour et al., 1995; Hurd, 1996; Peckys and Landwehrmeyer, 1999; Li et al., 2012). Notably, KORs expressed on mesocorticolimbic DA neurons play a key role in depressive-like behavior in rodents (Newton et al., 2002; Shirayama et al., 2004; Ebner et al., 2010; Muschamp et al., 2011b). KORs are expressed on both cell bodies and VTA terminals including those in the NAc (Svingos et al., 1999; Svingos et al., 2001) and activation of KORs inhibits neuronal activity

(Margolis et al., 2003; Margolis et al., 2006b; Ford et al., 2007). As such, KORs can regulate both the firing rates of DA neurons as well as DA release from terminals in efferent targets (Di Chiara and Imperato, 1988; Donzanti et al., 1992; Devine et al., 1993; Carlezon et al., 2006; Margolis et al., 2006b). Conversely, KOR antagonists increase extracellular concentrations of DA in the NAc (Maisonneuve et al., 1994) and direct injection of KOR antagonist into the NAc is sufficient to induce antidepressant-like effects (Newton et al., 2002; Shirayama et al., 2004), suggesting these effects are the result of alterations in DA function (Nestler and Carlezon, 2006). Although the VTA sends strong projections to the NAc, it also sends projections to areas such as the AMY and BNST, which are more directly implicated in fear and anxiety (Davis et al., 1997; LeDoux, 2000; Radke, 2009). While the role of the mesocorticolimbic DA system in anxiety-like behavior has not been thoroughly explored, the high comorbidity of depressive and anxiety disorders in humans (Kaufman and Charney, 2000; Kessler et al., 2003), suggests that this system may be involved in the pathology of both.

Here we examined anxiety-like behavior in two lines of KOR-mutant mice: one lacking KORs throughout the brain ($KOR^{-/-}$), and one lacking KORs specifically in DA neurons (DAT-$KOR^{lox/lox}$). These lines of mice were tested in two paradigms of stress-induced behavior—CRF-enhanced startle and footshock-potentiated startle—that were previously demonstrated to be sensitive to systemic KOR antagonism (Chapter 2 and 3). As a complementary approach to using DAT-$KOR^{lox/lox}$ mice, we ablated KORs in the VTA of adult floxed KOR ($KOR^{lox/lox}$) mice by infusing a viral vector expressing Cre into this region before footshock-potentiated startle testing. This approach enabled spatial (i.e., VTA) and temporal (i.e., during adulthood) restriction of KOR ablation.

Materials and Methods

Mice: $KOR^{lox/lox}$ and KOR-deficient mice were generated and genotyped as described previously (Chapter 4). Briefly, we generated mice in which exon 3 of the Oprk1 gene was flanked by loxP sites (inGenious Targeting Laboratory [iTL], Ronkonkoma, NY). These $KOR^{lox/lox}$ mice were then bred to Ella-Cre and DAT-Cre expressing mice to generate constitutive KOR KOs and conditional KOR KOs in which KORs are ablated from DA neurons respectively. Experiments were performed in 8-16-week-old male mice backcrossed for 7 generations to C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME). Mice were maintained on a 12:12-h light-dark cycle (0700h lights on) with *ad libitum* food and water available except during testing. Experiments were conducted during the light phase of the daily cycle. Experimental protocols were approved by the Institutional Animal Care and Use Committee of McLean Hospital and in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Academies Press, Washington D.C., USA, 2011) .

Drugs: JDTic was synthesized at Research Triangle Institute (Research Triangle Park, NC). Rat/human CRF was purchased from American Peptide (Sunnyvale, CA). JDTic was dissolved in 0.9% saline and administered IP at 10 mL/kg. CRF was dissolved in aCSF (Harvard Apparatus, Holliston, MA) and infused in a volume of 1.0 µL.

Stereotaxic surgery and microinfusions: Mice were anesthetized with ketamine/xylazine (100 mg/kg, 12.5 mg/kg, IP) and placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA) with zygoma ear cups in order to prevent damage to the ear drums. For each mouse, a stainless steel guide cannula (26-gauge, Plastics One,

Roanoke, VA) with a dummy stylet extending 1.5 mm beyond the end was lowered into the right lateral ventricle at the following coordinates, relative to bregma: anteroposterior = -0.2 mm, mediolateral = 1.0 mm, and lowered -2.4 mm ventral to the skull (Paxinos and Franklin, 2001). Non-acrylic cement (Geristore, Den-mat, Santa Maria, CA) permanently secured the cannula to the skull. The mice were allowed to recover for 5-7 days before testing. Drugs were microinfused by removing the dummy stylet and replacing it with a 33-gauge infusion stylet (Plastics One) attached to a Hamilton microsyringe (10 µl) by polyethylene tubing. ICV infusions of either vehicle (aCSF) or CRF (1.0 µg) were performed over a 2-min period at a rate of 0.5 µL/min, with an additional 2 min of diffusion time before the infusion stylet was removed and the dummy stylet was replaced. During the infusion mice were placed in clean and empty mouse cages divided in half and were free to move. After the infusion, mice were immediately placed in the startle chambers.

Viral-mediated gene transfer was used in some mice to ablate KORs within the VTA or adult mice. We used an adeno-associated virus (AAV) because it produces stable gene expression without notable toxicity (Ahmed et al., 2004); our AAV-GFP (control) and AAV-Cre/GFP vectors have been described previously (Berton et al., 2006; Graham et al., 2007) and were obtained from S.J. Russo, Mt. Sinai School of Medicine. $KOR^{lox/lox}$ mice received bilateral injections of AAV-GFP or AAV-Cre/GFP into the VTA at the following coordinates, relative to bregma: anteroposterior = -3.3 mm, mediolateral = ±0.5 mm, and lowered 4.1 mm ventral to the skull (Paxinos and Franklin, 2001) through a 27-gauge hypodermic tube attached to a Hamilton syringe (10 µl) by polyethylene tubing. Injections of 0.3 µL per side were made over a 3-min period. Injectors were left

in place for an additional 5 min before being slowly removed. The incision was closed with sutures.

Startle testing: The equipment and procedures used here were similar to those described in Chapters 2 and 3. Briefly, acoustic startle data were collected by measuring the amplitude of the startle reflex in response to white noise bursts of various intensities using the Med Associates Inc. (St. Albans, VT) Startle Reflex System and Advanced Startle software program. Mice were placed into 8.5 x 7 x 7 cm Plexiglas holders with steel rod floor bars attached to a load cell platform contained within a 40 x 64 x 42 cm sound-attenuating chamber. The load cell transduces movement into an electrical output that is amplified and digitized into arbitrary units by an analog-to-digital converter interfaced to a computer. Startle amplitude was defined as the maximum peak-to-peak voltage occurring within the first 100 ms after the onset of the startle stimulus. An audio stimulator generated 50 msec startle stimuli (1-32 kHz white noise, 1 msec rise-decay) which were delivered through high-frequency speakers located 4 cm behind the cages. The intensities of the startle stimuli were calibrated before use using customized software. All tests were conducted in darkness.

CRF-enhanced startle: Following recovery from surgery, mice were given a habituation session to acclimate them to the testing chamber. The habituation session consisted of a 5-min acclimation period followed by startle stimuli at 3 dB levels (20 each at 80, 90 and 100 dB) presented in a pseudo-random order with an ISI of 30 sec. Two days later, mice were infused with CRF (1.0 µg, ICV) and immediately placed in the startle chambers for a 5-min acclimation period followed by a 99-min startle test of 198 startle stimuli (80, 90, or 100 dB presented in a pseudo-random order with an ISI of 30 sec).

$KOR^{-/-}$ mice used to examine the effects of a KOR antagonist on CRF-enhanced startle received JDTic (30 mg/kg, IP) or vehicle (0.9% saline) immediately following surgery, and 7 d later were tested as above after administration of 1.0 µg CRF (ICV). The following day, mice were given another baseline startle session and 24 hr later were retested after administration of aCSF.

Footshock-potentiated startle: The footshock-potentiated startle test procedure was based on a prior report (Risbrough et al., 2009), with minor adaptations to the sound intensity (dB) levels, as described in Chapter 3. Briefly, mice were initially given a habituation startle session consisting of a 5-min acclimation period followed by 102 startle stimuli, 34 each at 80, 90, and 100 dB which occurred 24 hr before the test session. During the test session, mice received a 5-min acclimation period followed by 27 startle stimuli, 9 each at 80, 90, and 100 dB in a pseudo-random order with a 15 sec ISI to assess their baseline startle (startle test). Mice then received three sessions of 5 footshocks (shock session). The footshock intensity increased between sessions (i.e., 0.2 mA during session 1; 0.4 mA during session 2; and 0.8 mA during session 3). The average ISI between footshocks was 60 sec (30-90 sec). Mice received an additional startle test after each shock session to assess the magnitude of startle potentiation elicited by shock exposure. The following day, mice were given a final startle test to probe context conditioning in the absence of footshock. Mice that received viral vector infusions were tested in footshock-potentiated startle 30 d after surgery.

qPCR: We performed qPCR on cDNA synthesized from tissue punches of the VTA 30 d following intra-VTA infusion of AAV-Cre/GFP or AAV-GFP vector. Bilateral 1-mm^3

punches of VTA tissue were taken on a cryostat and kept on dry ice. RNA was extracted using PureLink RNA Mini Kit (Invitrogen). RNA quality and quantity were assessed using an RNA 6000 Nano Chip (Agilent, Santa Clara, CA) on an Agilent Bioanalyzer 2100. RNA integrity number (RIN) exceeded 7 for all samples, indicating high quality. 500 ng of total RNA was used to synthesize cDNA using iScript cDNA Synthesis Kit (BioRad) in a Thermo Hybaid iCycler (Thermo Scientific). Primers specific for KOR (*Oprk1*; 5-TCCTTGGAGGCACCAAAGTCAGGG-3, and 5-TGGTGATGCGGCGGAGATTTCG-3), DAT (*Slc6a3*; 5-AATGCCCTGGGCTGGATCATTGC-3 and 5-AATGGCGCAGCGTGAATTGGC-3), and integral membrane protein 2B (*Itm2b*; 5- CGCATCGAGAACGTGGACAACC-3 and 5-GTGGCATTGAATTCCTCCCCACG-3) genes were used. Melt curve analysis and polyacrylamide gel electrophoresis confirmed the specificity of the primers. The amplicon bp lengths are 237 (KOR), 178 (DAT), and 218 (ITM2B).

The qPCR was run using the iQ SybrGreen Supermix (BioRad). The reaction was carried out on a MyiQ Single Color Real-Time PCR Detection System (BioRad) in a volume of 20 µl, with 2.0 µL of 3 µM forward and reverse primers, 2.0 µL Rnase/Dnase free H_2O, 10 µL SybrGreen Supermix, and 4.0 µL cDNA sample diluted 1:10. PCR cycling conditions were 95°C for 5 min; 40 cycles at 94°C for 15 sec, 60°C for 15 sec, 72°C for 15 sec. Data were collected at a read temperature of 82°C for 15 sec. MyiQ Optical System Software (BioRad) was used to analyze the data, and reported values for KOR and DAT were normalized to ITM2B, which did not show group differences in qPCR analyses. All samples were run in triplicate.

Histological verification: Mice were overdosed with pentobarbital (130 mg/kg, IP) and transcardially perfused with 0.9% saline (20 mL) followed by 4% paraformaldehyde in 0.1M PBS (60 mL). Brains were removed and postfixed overnight in 4% paraformaldehyde, and then cryoprotected in 30% sucrose/0.1M PBS for 24 h. Brains were sectioned at 30 µm on a freezing microtome and collected in 0.1M phosphate buffer. Sections were mounted on gelatin-coated slides (Fisher Scientific, Pittsburg, PA) and stained with 0.1% cresyl violet. For AAV infused brains, the region of GFP expression was determined with a fluorescence microscope. Mice with incorrect placements or signs of infection were excluded from analysis.

Data analysis: The effect of genotype on CRF-enhanced startle was analyzed with a 3-way (genotype x CRF x dB level) ANOVA with repeated measures (dB and CRF). Footshock-potentiated startle was analyzed by 3-way ANOVA (genotype x block x dB level) in shocked mice. Significant interactions in the ANOVAs were further analyzed using Bonferroni *post hoc* tests.

Results

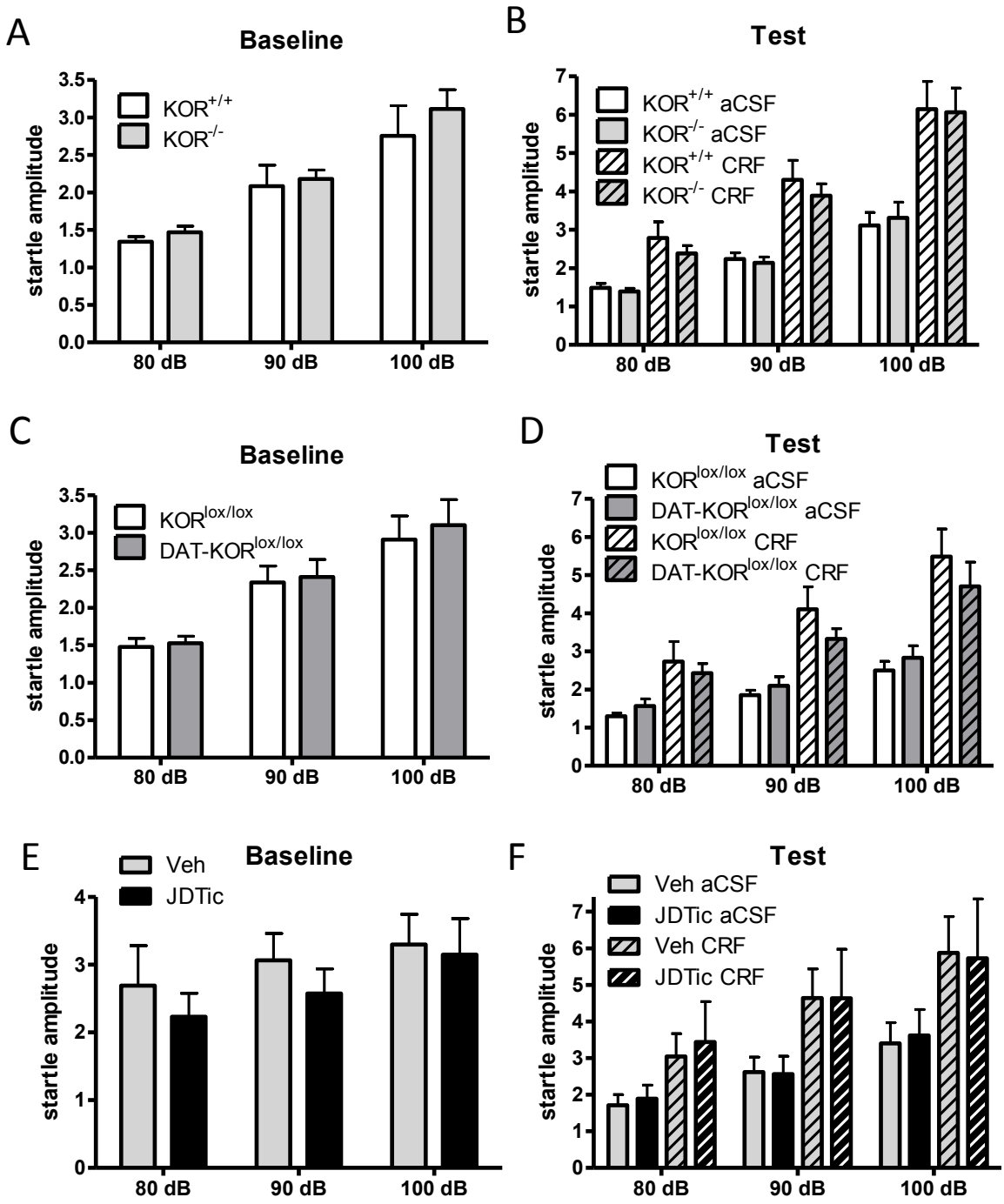
Following recovery from surgery, mice lacking KORs constitutively ($KOR^{-/-}$) or specifically in DA neurons (DAT-$KOR^{lox/lox}$) were tested in CRF-enhanced startle. Baseline startle amplitudes did not differ between $KOR^{-/-}$ mice and $KOR^{+/+}$ littermate controls (main effect of dB, $F[2,56]=40.97$, $P<0.0001$; **Fig. 5.1A**). CRF-infusion significantly increased startle reactivity in both genotypes ($F[1,28]=34.87$, $P<0.0001$) and there was not a genotype x CRF interaction observed, indicating that the mutant mice displayed normal CRF-enhanced startle (**Fig. 5.1B**). Likewise, baseline startle responding was dependent on dB level ($F[2,70]=45.00$, $P<0.0001$), but did not differ

between DAT-KOR$^{lox/lox}$ and littermate controls (**Fig. 5.1C**). As was the case with the constitutive mutants, CRF significantly increased startle responding (F[1,35]=29.22, *P*<0.0001) that was not dependent on genotype in the conditional mutant line (**Fig. 5.1D**), although KOR ablation did tend to cause nominal reductions in CRF effects (F[1,35]=1.90,NS).

To determine if the ability of JDTic to block CRF-enhanced startle (Chapter 2) could be due to off-target effects unrelated to KOR blockade, KOR$^{-/-}$ mice were pretreated with the drug (30 mg/kg, IP) 7 days before a CRF-enhanced startle test, to match the time course of previous work (see Chapter 2). The following day they were given another baseline startle test session and 24 hr later were infused with aCSF and tested again. Vehicle- and JDTic-treated groups did not differ in baseline startle responding following IP treatment (i.e., before CRF treatment) (main effect of dB, F[2,38]=7.28, *P*<0.01; **Fig. 5.1E**). In the test sessions, CRF significantly increased the acoustic startle reflex independent of JDTic treatment (F[1,19]=14.15, *P*<0.001; **Fig. 5.1F**), suggesting that JDTic effects observed previously were the result of on-target (KOR-specific antagonism) effects of the drug.

Figure 5.1 Effect of KOR ablation on CRF-enhanced startle. During habituation baseline startle reactivity was compared. ***A.*** No genotype effects were observed between $KOR^{-/-}$ and littermate controls ($KOR^{+/+}$). ***B.*** On the test day, mice received CRF (1.0 µg) or vehicle and were immediately placed in the startle apparatus. Constitutive KOR ablation did not affect CRF-induced increases in startle reactivity (n=14-16). ***C.*** DAT-$KOR^{lox/lox}$ mice had equivalent levels of startle reactivity as $KOR^{lox/lox}$ controls (n=17-20). ***D.*** Ablation of KORs from DA neurons nominally attenuated, but did not significantly reduce CRF-enhanced startle. ***E.*** Baseline startle reactivity was not affected by JDTic (30 mg/kg, IP) pretreatment in $KOR^{-/-}$ mice (n=10-11). ***F.*** JDTic did not significantly alter CRF-induced increases in startle amplitude at any dB level in $KOR^{-/-}$ mice. Data were analyzed by ANOVA.

Figure 5.1 (Continued) Effect of KOR ablation on CRF-enhanced startle



The two lines of KO mice were next tested in footshock-potentiated startle, a paradigm in which systemic KOR antagonism was also found to have an anxiolytic-like effect (Chapter 3). Consistent with the results reported in Fig. 5.1 above, $KOR^{+/+}$ and $KOR^{-/-}$ did not differ in baseline startle across the 3 startle intensities used (80, 90, and 100) immediately preceding footshock presentation (main effect of dB, $F[2,36]=15.89$, $P<0.0001$; **Fig. 5.2A**). After this baseline session, mice received footshocks of increasing intensity (0.2, 0.4 and 0.8 mA) each followed by a startle session that was compared to the baseline session to calculate percent potentiation. Both genotypes showed similar reactivity during footshock presentation (**Table 1**), with increasing footshock amplitude increasing this response ($F[2,46]=132.03$, $P<0.0001$). Footshock-potentiated startle depended on block ($F[2,46]=6.51$, $P<0.01$), but did not differ between $KOR^{-/-}$ mice and littermate controls (**Fig. 5.2D**). When mice were re-tested in the context 24 hr later, they still displayed increased startle reactivity above baseline and genotype did not affect this response (**Fig. 5.2G**).

DAT-$KOR^{lox/lox}$ and $KOR^{lox/lox}$ controls began the test session with similar baseline startle responding (main effect of dB, $F[2,36]=15.89$, $P<0.0001$; **Fig. 5.2B**). Likewise, they were not significantly different in their response during presentation of footshock (**Table 1**). However, in the test session, footshock-potentiated startle depended on a genotype x block interaction ($F[2,36]=9.72$, $P<0.05$). *Post hoc* tests revealed that DAT-$KOR^{lox/lox}$ mice had significantly less footshock-potentiated startle following 0.2 mA and 0.4 mA footshock blocks compared to littermate controls (*P*'s<0.05), but were virtually identical after 0.8 mA footshocks (**Fig. 5.2E**). A follow-up contextual conditioning test conducted 24 hr later did not reveal any differences between DAT-$KOR^{lox/lox}$ mice and controls

(**Fig. 5.2H**) indicating similar levels of anxiety-like behavior when returned to the footshock context. Mice expressing DAT-Cre, but lacking floxed KOR alleles (DAT-$KOR^{+/+}$) mice and controls ($KOR^{+/+}$) mice were tested to ensure that decreases in footshock-potentiated startle were not simply due to Cre expression alone. DAT-Cre did not affect baseline startle (main effect of dB, $F[2,40]=49.59$, $P<0.0001$; **Fig. 5.2C**) or footshock reactivity (**Table 1**). Analysis of potentiation following footshock did not reveal any significant differences between DAT-$KOR^{+/+}$ mice and $KOR^{+/+}$ littermate controls (**Fig. 5.2F**). Further, no differences were observed during the context test 24 hr after footshock-potentiated startle (**Fig. 5.2I**).

Figure 5.2 Effect of KOR ablation on footshock-potentiated startle. ***A-C.*** Baseline startle reactivity in mutant and control mice revealed no genotype differences. ***D.*** Constitutive KOR deletion did not affect footshock-potentiated startle (n=8-9). ***E.*** DAT-$KOR^{lox/lox}$ mice had significantly reduced potentiation following 0.2 and 0.4 mA blocks of footshock compared to $KOR^{lox/lox}$ controls (n=10). ***F.*** Reductions in footshock-potentiated startle observed in DAT-$KOR^{lox/lox}$ mice were not due to DAT-Cre expression alone (n=11). ***G-I.*** When mutant mice were re-tested in the footshock context 24 hr later, they were indistinguishable from littermate controls. Data were analyzed by ANOVA followed by Bonferroni *post* test as appropriate. * $p<0.05$

Figure 5.2 (Continued) Effect of KOR ablation on footshock-potentiated startle

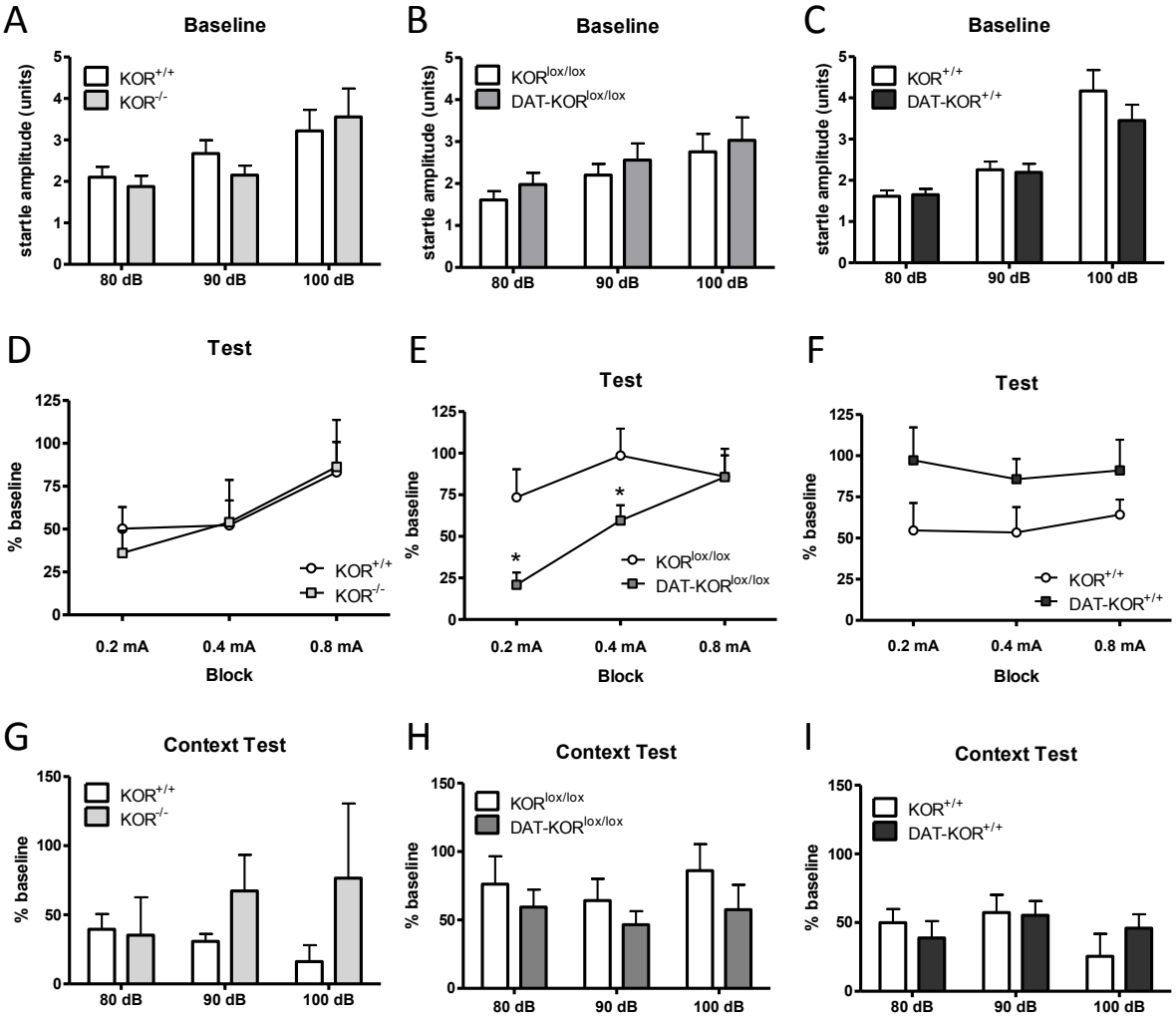


Table 5.1 Footshock reactivity in KOR-deficient mice

			$KOR^{lox/lox}$		$KOR^{+/+}$	
mA	$KOR^{+/+}$	$KOR^{-/-}$	DATCre-	DATCre+	DATCre-	DATCre+
0.2	7.23 ± 1.13	7.23 ± 1.19	7.75 ± 1.00	6.27 ± 1.18	8.69 ± 1.52	8.52 ± 1.39
0.4	16.99 ± 0.65	14.37 ± 2.15	16.49 ± 0.78	15.14 ± 1.13	16.75 ± 0.71	16.44 ± 0.92
0.8	17.94 ± 0.57	18.04 ± 0.57	17.74 ± 0.56	17.30 ± 0.70	17.69 ± 0.69	16.08 ± 0.84

Measures reported as mean ± SEM of average reactivity during the presentation of footshock. No significant effects of genotype were observed.

To complement the conditional KO studies, AAV-Cre/GFP was used to ablate KORs from the VTA of $KOR^{lox/lox}$. This technique allows for spatial- (i.e., within VTA) and temporal- (i.e., during adulthood) specific ablation of KORs in the same system affected in the DAT-$KOR^{lox/lox}$ line of mice. GFP expression was localized to the VTA when examined 30 d after AAV infusion—the time point at which our behavioral studies were conducted (**Fig. 5.3A**). Further, mRNA levels of the KOR gene were significantly reduced in mice receiving Cre vector compared to GFP vector controls ($P<0.05$) whereas DAT expression was unaffected, suggesting specific Cre-induced reductions (**Fig. 5.3B&C**). Cre expression did not affect baseline startle (main effect of dB, $F[2,42]=34.73$, $P<0.0001$; **Fig. 5.3D**) or shock reactivity (**Table 2**). Footshock-potentiated startle was not significantly affected by Cre-mediated KOR gene deletion in the VTA (**Fig. 5.3E**) and mice did not differ when re-tested 24 hr later in the footshock context (**Fig. 5.3F**).

Figure 5.3 Effect of intra-VTA AAV-Cre/GFP on footshock-potentiated startle in KOR$^{lox/lox}$ mice. ***A.*** AAV-Cre/GFP or control vector (AAV-GFP) was infused into the VTA of adult KOR$^{lox/lox}$ mice and GFP fluorescence was visualized 30 d later to confirm injection site. ***B.*** VTA punches were taken from untested AAV infused mice 30 d following infusion and processed for qPCR. mRNA levels of the KOR gene (*OPRK1*) were significantly reduced in KOR$^{lox/lox}$ mice receiving AAV-Cre/GFP compared to GFP control vector. ***C.*** Levels of DAT (*Slc6a3)* mRNA were equivalent between groups. ***D.*** In footshock-potentiated startle experiments, mice were infused with viral vector and allowed to recover for 28 d. Baseline startle reactivity was tested on day 29 and did not differ between AAV-Cre/GFP and AAV-GFP infused mice (n=11-12). ***E,F.*** Mice infused with AAV-Cre/GFP were not significantly different from controls in footshock-potentiated startle 30 d after surgery or when re-tested 24 hr following footshock. Data were analyzed by ANOVA or Student's t-test as appropriate.

Figure 5.3 (Continued) Effect of intra-VTA AAV-Cre/GFP on footshock-potentiated startle in KOR$^{lox/lox}$ mice.

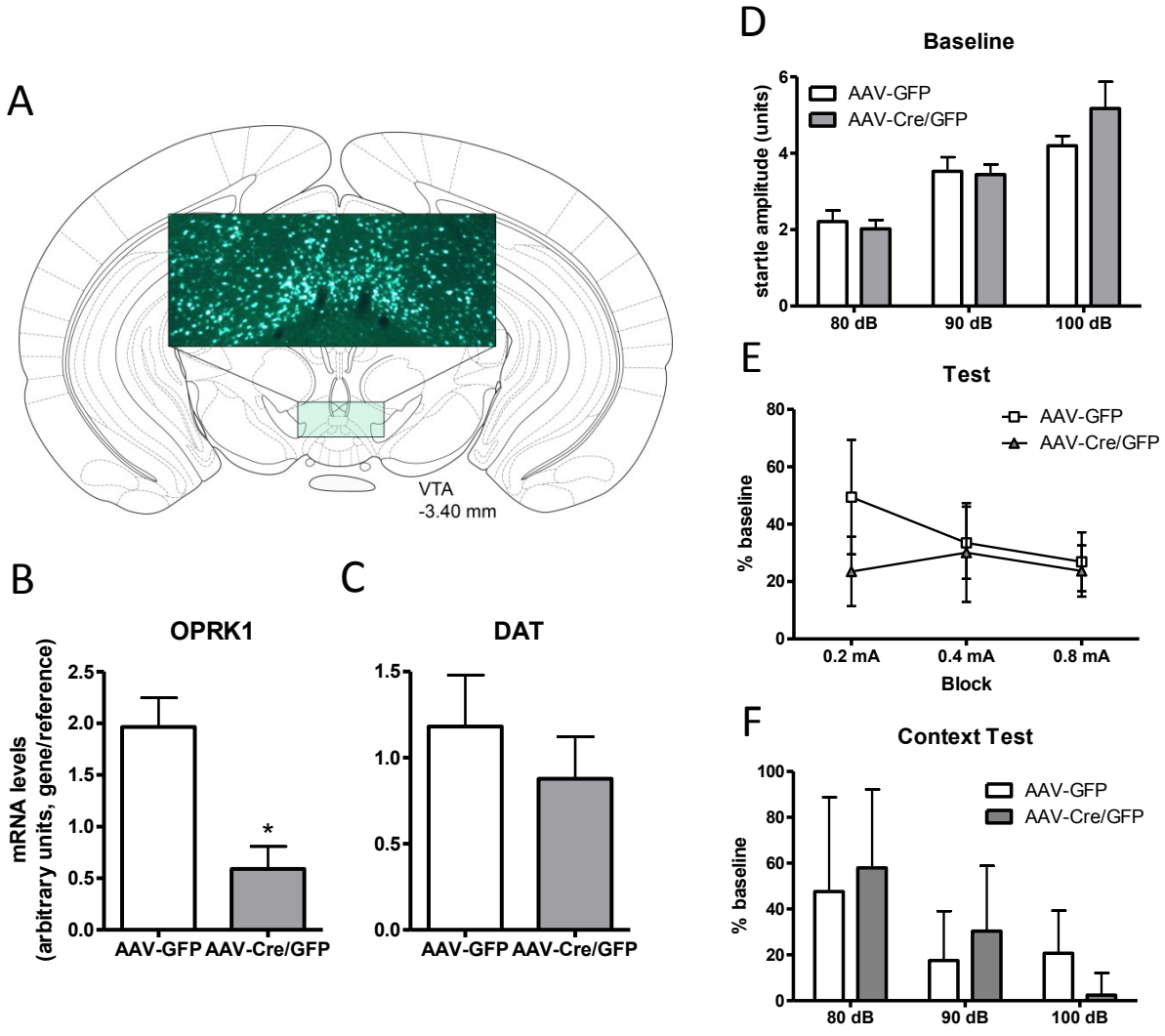


Table 5.2 Footshock reactivity in AAV-infused KOR$^{lox/lox}$ mice

mA	AAV-GFP	AAV-Cre/GFP
0.2	8.67 ± 1.40	8.83 ± 1.99
0.4	15.65 ± 1.75	14.71 ± 2.53
0.8	16.38 ± 0.68	18.32 ± 1.85

Measures reported as mean ± SEM of average reactivity during the presentation of footshock. No significant effects of viral vector were observed.

Discussion

Ablation of KORs specifically in DA neurons produced anxiolytic-like effects in footshock-potentiated startle, but similar levels of potentiation following ICV CRF compared to controls. Neither startle measure was affected by constitutive KOR deletion, suggesting greater compensation in KOR function during development in these KOs compared to conditional KOs. Ablation of KORs in the VTA using AAV-Cre/GFP viral vector microinfused into $KOR^{lox/lox}$ mice was not sufficient to reproduce the attenuation of footshock-potentiated startle observed in DAT-$KOR^{lox/lox}$ mice. These findings provide support for a role of mesolimbic KORs in the etiology of stress-induced anxiety responses under some—but not all—experimental conditions.

We first investigated the role of KOR function in the mutant mice following infusion of the anxiogenic peptide CRF. Neither constitutive nor conditional ablation of KORs affected the ability of CRF to enhance startle. This was unexpected given our previous data demonstrating a significant attenuation of CRF-enhanced startle by systemic administration of the long-lasting and highly selective KOR antagonist JDTic (Chapter 2). There was a nominal reduction of CRF effects in the DAT-$KOR^{lox/lox}$ mice at the highest startle intensities. This effect was not statistically significant despite the use of comparable group sizes as in the JDTic studies, which might indicate that the effects of the mutation are weaker than those of pharmacological blockade, or that JDTic effects are due to actions at receptors other than KORs. To determine if the effects of JDTic in WT mice might be due to off-target effects unrelated to KORs, we examined the effects of the drug on CRF-enhanced startle in $KOR^{-/-}$ mice. Importantly, JDTic failed to affect CRF-enhanced startle in $KOR^{-/-}$ mice, indicating that its ability to reduce CRF effects in

WT mice is due to specific blockade of KORs. Therefore, the dissociation between genetic and drug studies might be explained by differences between the effects of KOR ablation throughout development and acute pharmacological KOR blockade during adulthood. As one example, the neural systems that regulate anxiety-like behaviors (Lin et al., 2006; Marchant et al., 2007; Durant et al., 2010) may undergo developmental adaptations that can compensate for KOR ablation. Hence, behavioral phenotypes that are seen with pharmacological blockade in a normally-developing animal may be masked in constitutive KOR mutant mice.

Although $KOR^{-/-}$ mice were indistinguishable from $KOR^{+/+}$ controls in footshock-potentiated startle, DAT-$KOR^{lox/lox}$ mice demonstrated reduced potentiation following footshock compared to $KOR^{lox/lox}$ controls. This reduction was significant proceeding the first two blocks of footshock, but not in the final block. In our study design, the footshock amplitude in the test progressively increases from 0.2 to 0.4 to 0.8 mA. Thus KOR ablation from DA neurons was sufficient to reduce potentiation of startle in response to mild-to-moderate footshock stress, but not effective at the highest intensity of footshock tested. This may explain, at least in part, why startle responses in DAT-$KOR^{lox/lox}$ mice were not significantly different from those of controls in the CRF-enhanced startle studies. The intensity of stress following 1.0 µg CRF infusion may be greater than or at least equivalent to the stressful effects of 0.8 mA footshocks that likewise were not augmented in DAT-$KOR^{lox/lox}$ mice. Potential evidence for this stressor ceiling effect comes from c-Fos activation studies in Chapters 2 and 3. While 1.0 µg of CRF robustly increased c-Fos cell counts in all regions analyzed, a footshock session using 0.2 and 0.4 mA footshocks failed to do so. Further experiments in which

DAT-KOR$^{lox/lox}$ mice are infused with a lower dose of CRF (e.g. 0.5 µg) may reveal whether this less stressful condition can be overcome in conditional KO mice. Our c-Fos expression data and others (Bittencourt and Sawchenko, 2000; Warnock et al., 2009) also suggest that CRF given ICV is able to activate CRF-Rs throughout the brain. Thus if anxiolytic-like effects of KOR ablation in DA neurons were the result of decreases in CRF release and CRF-R activation, central infusions of CRF would negate these effects by diffusing to and directly activating these receptors. If this hypothesis is true, we may expect DAT-KOR$^{lox/lox}$ mice to have decreases in mild footshock stress-induced CRF release in brain regions such as the BNST—into which direct infusions of CRF mimic ICV effects (Liang et al., 1992) —compared to KOR$^{lox/lox}$ controls.

As an alternative to using a breeding strategy to ablate KORs in DAT-expressing neurons—which offers spatial but not temporal specificity—we used an AAV-Cre/GFP viral vector to specifically ablate KORs in the VTA of adult KOR$^{lox/lox}$ mice. This approach ensures normal KOR expression during development and thus eliminates compensatory changes that may occur when KORs are ablated during this period. Infusions of the Cre-expressing vector did not have a significant effect on footshock-potentiated startle although they produced a slight but nonsignificant reduction following the first block of shock, reminiscent of the effect seen in DAT-KOR$^{lox/lox}$ mice. There are several possibilities as to why Cre viral vector manipulations do not replicate findings in DAT-KOR$^{lox/lox}$ mice. One possibility is that whereas KOR ablation in DAT-KOR$^{lox/lox}$ mice is restricted to DAT-expressing cells, KOR ablation produced by the Cre vector occurs in all infected neurons, regardless of type. Although the VTA predominantly consists of DA neurons, there are also populations of GABAergic and glutamatergic

neurons within this region (Margolis et al., 2006a), any of which may express KORs. Further, these non-DA VTA cells typically project to the same structures as DA neurons (Swanson, 1982). Because $KOR^{lox/lox}$ mice were used in combination with a viral vector that infects all cell types, KOR expression is ablated at the injection site non-specifically; considering that KORs are in presynaptic terminals where they can inhibit GABA and glutamate release (Hjelmstad and Fields, 2003; Li et al., 2012), ablation of KORs in both DA and non-DA neurons might have counteracting effects. Use of electrophysiological and optogenetic approaches targeting non-DA cells in the VTA in future studies may elucidate whether KOR activation in this population opposes KOR activation effects in DA cells. A second possibility is that although KOR mRNA is significantly reduced 30 d following Cre infusion, receptors produced before viral vector infusion may still persist at the cell membrane. Studies using a nonreversible KOR agonist suggest that KOR turnover requires at least 14 days (McLaughlin et al., 2004). As proteins are capable of lasting for weeks before degradation (Creighton, 1993), it is possible that a reserve of KORs at insertion sites may be sufficient to repopulate through the time of testing. This possibility can be addressed in future studies by determining whether binding of [^{3}H]U69,593, a tritiated version of a highly selective KOR agonist is lower in the VTA of AAV-Cre/GFP viral vector infused mice compared to AAV-GFP viral vector controls 30 d following infusion. A third possibility is that attenuation of responding may have been missed because of a reduced range of potentiation. Mice infused with control virus displayed only modest potentiation following the 2nd and 3rd block of footshock (startle increases of less than 30% over baseline), in contrast to earlier non-virus infused mice whose potentiation was over 50% and as high as 100%. This reduced potentiation might be the result of increases in baseline startle in mice receiving VTA infusions; in

fact, we found that startle reactivity was significantly higher in control GFP vector mice compared to no-surgery group controls from the studies presented here (data not shown). Because the startle response was not quantified before surgery, it is unclear whether these effects were due to microinfusion or strain differences. Surgery may also have disrupted learning processes that account for the minimal conditioning responses. While VTA lesions do not affect baseline startle, they have been shown to disrupt increases in startle to fear-eliciting cues (Borowski and Kokkinidis, 1996). Damage to the VTA during surgery by the injection cannula or the hydrolic pressure of the infusion may account for the nominal increases in startle following footshock in both control and experimental groups. This possibility could be addressed in future studies by using a delivery system (such as pulled-glass micropipettes) with a smaller diameter together with slower infusion volumes and/or rates. Yet another possibility is that the effects observed in the DAT-KOR$^{lox/lox}$ conditional KO mice may be due to DAT-expressing neurons outside of the VTA, for example in the SN. Further work using Cre viral vectors to ablate KORs in non-VTA DA populations may clarify whether KORs in these regions are necessary for stress-induced increases in anxiety-like behavior. Finally, the effects observed in the DAT-KOR$^{lox/lox}$ conditional KO mice may be unrelated to a lack of KOR signaling in adulthood, and instead be a product of developmental changes that occur due to the absence of KORs in DA neurons. In this scenario, ablating KORs from the VTA in adulthood would have no effect since development occurred normally. Experiments in which VTA KORs are rescued during adulthood in DAT-KOR$^{lox/lox}$ mice may help to determine if KOR disruption alone is sufficient to attenuate footshock-effects.

In the present studies, mice lacking KORs in midbrain DA neurons throughout their life exhibited significant decreases in footshock-potentiated startle. This work confirms KOR antagonist and dynorphin KO mouse studies that indicate a role for KOR signaling in stress-sensitized behaviors (Knoll et al., 2007; Wittmann et al., 2009; Carr and Lucki, 2010) and implicates KORs on DA neurons in these effects. The effect was surprisingly weak, considering the effects of the KOR agonist JDTic, and not recapitulated by viral vector-induced ablation of KORs within the same region. Although the viral vector studies have numerous important qualifications and limitations, the pattern of results observed raises the possibility that KOR blockade in regions not studied in this project also play an important role in regulating signs of anxiety that are reflected by the ASR. Although beyond the scope of this project, candidate regions for future studies of KOR ablation include the AMY (Knoll et al., 2011), BNST (Li et al., 2012), and PFC (Margolis et al., 2006b). In addition, future studies to delineate the specific dopaminergic projections augmented by KOR activation may also provide valuable insight into the specific function of neural circuits in anxiety-like states.

Chapter 6

Conclusions

The underlying neurobiological mechanisms of anxiety disorders are not understood. Severe or prolonged stress often coincides with the development of these disorders (Kessler, 1997; Heim and Nemeroff, 1999), suggesting that CRF, a key mediator of stress effects in the brain, may mediate these responses. Indeed, dysfunction of the CRF system is thought to underlie at least some forms of pathological anxiety (Nemeroff, 1992; Owens et al., 1993; De Souza, 1995). The KOR system has also been implicated in the negative consequences of stress and CRF (Pliakas et al., 2001; Newton et al., 2002; Mague et al., 2003; McLaughlin et al., 2003a; McLaughlin et al., 2006b; Land et al., 2008; Carey et al., 2009; Land et al., 2009) making it a promising target for therapeutic intervention. Previous work has demonstrated a role for KORs in the mesocorticolimbic DA system in depressive-like behavior (Newton et al., 2002; Shirayama et al., 2004; Muschamp et al., 2011b), and because anxiety disorders are highly comorbid with depression (Kaufman and Charney, 2000; Kessler et al., 2003), we hypothesized that this system may be an important site of KOR activation in the development of anxiety-like behavior as well. My dissertation work investigated the general role of KORs in mouse models of stress-induced behavior and tested the hypothesis that KORs within the mesocorticolimbic DA system play a particularly important role in regulating anxiety-related behavior.

1. Summary of findings

CRF plays a major role in the regulation of the stress response and reproduces the behavioral, hormonal, and endocrine consequences of stress. Disrupting KOR function can block effects of CRF, suggesting KOR activation plays an important role in stress-induced effects (Land et al., 2008; Bruchas et al., 2009). I first sought to determine if KOR antagonism could block anxiety-like effects of CRF by examining enhancement of the ASR following CRF infusion. I found that central infusions of CRF dose-dependently enhanced the acoustic startle response in mice, whereas, pretreatment with the KOR antagonist JDTic significantly attenuated this effect. This was not due to non-specific reductions in the capacity to respond, because JDTic treated mice had equivalent levels of baseline startle as controls. To identify brain regions that may be involved in the effects of JDTic, I quantified c-Fos expression following CRF infusion within areas implicated in depressive and anxiety-like behaviors. While c-Fos immunoreactivity in the dDG was decreased in JDTic mice regardless of whether mice received infusions of CRF or vehicle, it was specifically reduced in the VTA of JDTic mice following CRF. This reduction was due, at least in part, to decreased activation of DA cells in the VTA: the percent of co-labeled c-Fos and TH cells was significantly lower following JDTic than following vehicle. All of the other regions studied had increased c-Fos expression in CRF compared to aCSF-infused brains, but there were no further effects of JDTic. These studies suggest that KORs may alter the function of the mesocorticolimbic system to produce beneficial effects on anxiety-like behavior.

The ASR can also be potentiated by brief presentation of mild footshock immediately before testing (Davis, 1989). This behavior likely depends on CRF function, because

mice lacking CRF receptors lack footshock-potentiated startle (Risbrough et al., 2009). To determine if KOR antagonism would also block footshock effects on startle, I systemically administered JDTic proceeding footshock-potentiated startle testing. As was the case in our CRF studies, JDTic produced anxiolytic-like effects in footshock-potentiated startle without affecting baseline reactivity. I again performed a histochemical analysis of c-Fos immunoreactivity to compare brain activation patterns between JDTic-treated mice subjected to footshock and controls. Footshock induced an increase in c-Fos expression in the PVN of control mice; however, no differences were observed in any region of the HIP or VTA, areas in which footshock-induced increases in c-Fos expression have been reported (Smith et al., 1992; Funk et al., 2003; Funk et al., 2006). Thus I could not establish any associations between behavioral endpoints and neural activation in the brain regions studied. Nevertheless, my behavioral data confirm that KOR antagonists can produce anxiolytic-like effects following a single injection. This profile may make them attractive treatments for anxiety disorders or preventing the effects of stress when exposure can be predicted.

To complement the work with KOR antagonists, we developed a line of floxed KOR ($KOR^{lox/lox}$) mice to enable spatial and, ultimately, temporal control of receptor ablation. These floxed mice were bred to two Cre-expressing mouse lines (Ella-Cre and DAT-Cre) to generate constitutive ($KOR^{-/-}$) mutants and conditional (DAT-$KOR^{lox/lox}$) mutants in which KORs are ablated in DA neurons. While other lines of $KOR^{-/-}$ mice exist, to our knowledge this is the first thorough characterization of a floxed KOR mutant and lines derived from such. Radioligand studies quantifying tritiated KOR agonist binding in mutant mouse brains confirmed the absence of functional KORs in $KOR^{-/-}$ mice and

reductions in functional KORs in the VTA and its efferents in DAT-KOR$^{lox/lox}$ mice. Further, brain region-specific qPCR confirmed the absence of KOR mRNA throughout the brain in KOR$^{-/-}$ mice, and reductions within the midbrain (comprising VTA and SNc) of DAT-KOR$^{lox/lox}$ mice. Neither line was significantly different than controls in metrics quantifying weight gain, vision, hearing, or locomotor activity. Considering previous work demonstrating a role for KORs in anxiety-like behavior both here (Chapters 2 and 3) and by others (Knoll et al., 2007; Bruchas et al., 2009; Wiley et al., 2009; Wittmann et al., 2009; Knoll et al., 2011), we next subjected our KOR mutant mice to tests of exploratory anxiety-like behavior. Whereas KOR$^{-/-}$ mice behaved as WTs in the open field, DAT-KOR$^{lox/lox}$ mice displayed an anxiolytic-like phenotype, spending significantly more time than controls in the center area of the enclosure in the later parts of the test session. A separate cohort of these mice was then tested in the light/dark box and had significantly shorter latencies to enter the light compartment, although this same cohort behaved normally when subsequently tested in the EPM. These effects are broadly consistent with previously reported anxiolytic-like effects of KOR antagonists, suggesting that pharmacological and genetic disruption of KOR function can produce similar phenotypes under some conditions.

We also investigated the effects of cocaine-induced locomotor sensitization in our mutant lines, to determine if they would replicate prior work demonstrating a pre-sensitized phenotype in a different line of KOR$^{-/-}$ mice (Chefer et al., 2005). We treated mice with IP injections of cocaine daily from days 1-5 and then again on day 8. Surprisingly, our line of KOR$^{-/-}$ mice had equivalent sensitization to KOR$^{+/+}$ littermates. The inconsistency of these data with findings reported by other groups may be due to

strain differences or flanking gene effects (see Chapter 5 discussion). However, DAT-KOR$^{lox/lox}$ mice appeared similar to controls after the first injection of cocaine, but in subsequent days displayed a heightened response to cocaine effects on locomotor activity and were significantly different from controls on day 8 of testing. Additional experiments indicated that this was not due to non-specific effects of Cre expression, because the behavior of mice expressing Cre but not the floxed KOR gene did not differ from that of controls. To complement these studies evaluating sensitivity to the stimulant effects of cocaine, we performed CPP studies to determine if DAT-KOR$^{lox/lox}$ mice would also have increased sensitivity to the rewarding effects of cocaine. Neither DAT-KOR$^{lox/lox}$ mice nor KOR$^{lox/lox}$ controls developed CPPs at a sub-threshold dose of cocaine, and both developed equivalent CPPs at an effective dose of the drug (Horger et al., 1999). These data are consistent with other work showing no differences in cocaine-induced CPP in either KOR or dynorphin KO mice compared to controls (McLaughlin et al., 2003a; McLaughlin et al., 2006a; Redila and Chavkin, 2008). Although sensitization to the locomotor-stimulating effects of drugs such as cocaine in rodents is sometimes used as a proxy for drug reward or drug seeking—an approach fraught with conceptual and practical flaws (Sutton and Self, 2000; Ahmed and Cador, 2006)—the differential effects seen here are likely due to differences in neural processes underlying each behavior. Additionally, 2 days of cocaine administration in the CPP studies may not be sufficient to reveal mutation-induced differences in drug sensitivity, considering that 6 days of cocaine administration were required to reveal significant differences in locomotor sensitization. Further work investigating CPP in mice that receive more drug exposure may clarify whether alternative cocaine treatment protocols might induce adaptations that are dependent on the KOR system in DA

neurons. Regardless, this work establishes an association between KORs expressed on DA neurons and the development of an important form of cocaine-induced neuroplasticity.

I then used the CRF-enhanced and footshock-potentiated startle paradigms to determine if genetic KOR ablation would affect stress-induced effects on startle. $KOR^{-/-}$ mice had normal levels of enhanced startle in response to both CRF infusion and footshock and were not significantly different from $KOR^{+/+}$ littermates in either paradigm. In contrast, whereas the behavior of DAT-$KOR^{lox/lox}$ mice was normal in the CRF-enhanced startle test, they had reduced footshock-potentiated startle. This potentiation was significantly lower than controls following 0.2 and 0.4 mA footshock blocks, although mutant and control mice had nearly equivalent potentiation during the 0.8 mA footshock block. To complement studies with DAT-$KOR^{lox/lox}$ mice, in which KORs are absent in DA neurons, I infused an AAV-Cre/GFP viral vector into the VTA of adult $KOR^{lox/lox}$ mice to specifically ablate KORs within this region. Surprisingly, the mice were not statistically different from controls in footshock-potentiated startle; however, there are several explanations as to why $KOR^{lox/lox}$ mice given intra-VTA infusions of Cre viral vector do not replicate conditional KO results (See discussion in Chapter 5). Overall, these data implicate KORs in DA neurons in the manifestation of stress-induced behaviors such as footshock-potentiated startle, but suggest KORs outside of the DA system may also play an important role in regulating signs of anxiety that are reflected by increases in the ASR.

In total, the work presented in my thesis confirms a role for KORs in the expression of anxiety-like behavior in rodents and for the first time suggests that dopaminergic neurons are notably involved in some of these effects. These suggest common neuroanatomic substrates for depressive and anxiety disorders, and raise the possibility that KOR antagonists would be useful for treating these disorders, particularly when they are comorbid.

2. CRF and KOR interactions in the dDG

In studies examining CRF-induced c-Fos expression, JDTic pretreatment significantly reduced c-Fos labeling in both the VTA and dDG of the HIP. In further work we chose to focus on the VTA; however, decreases in HIP activity may be an important component of the mechanism through which systemic JDTic reduces anxiety-like behavior. The HIP is positioned to modulate responses to stress and has reciprocal connections with key components of fear and anxiety circuitry including the amygdala (Pitkanen et al., 2000) and is thus capable of influencing fear and anxiety behavior. Of all brain areas responsive to stress, the HIP appears most vulnerable to its damaging effects (McEwen and Magarinos, 2001). Both acute and chronic stress models have been shown to decrease hippocampal neurogenesis in lab animals (Gould et al., 1997; Gould et al., 1998; Tanapat et al., 2001), a process implicated in learning and memory (Neves et al., 2008). Further, smaller HIP volumes are seen in PTSD sufferers (Bremner et al., 1995; Gurvits et al., 1996; Bremner et al., 1997b; Stein et al., 1997; Gilbertson et al., 2002; Villarreal et al., 2002; Bremner et al., 2003; Wignall et al., 2004; Bossini et al., 2008) and HIP size correlates with trauma severity such as combat exposure (Gurvits et al., 1996) and symptom severity (Stein et al., 1997; Villarreal et al.,

2002; Bremner et al., 2003). It is unclear when these changes in volume take place, though one twin study suggests smaller HIP volumes may occur before trauma and be a risk factor for pathology (Gilbertson et al., 2002). Regardless, these maladaptive differences in PTSD patients may underlie HIP dysfunction and promote fear and anxiety-like behavior.

Preclinical studies demonstrate that the HIP is involved in unconditioned anxiety-like responses as lesions of the HIP or inhibition of dorsal HIP function using the indirect $GABA_A$ agonist midazolam or the direct agonist muscimol produce anxiolytic-like effects in the EPM and social interaction tests (Gonzalez et al., 1998; Menard and Treit, 2001; Bannerman et al., 2002; Rezayat et al., 2005).. Studies also suggest a role for the HIP in contextual conditioning—anxiety-like behavior induced by the environment where an unconditioned stimulus (e.g. footshock) was presented. In particular, IEG products with transcriptional activity, such as c-Fos, may link brief context exposures to cellular changes resulting in memory formation (Davis and Squire, 1984; Tischmeyer and Grimm, 1999). Recently, an elegant study demonstrated that activation of a specific population of conditioning-responsive neurons (expressing c-Fos during conditioning) in the HIP was sufficient to induce anxiety-like behavior outside of the conditioned context (Liu et al., 2012). These finding suggest that the HIP plays a role in anxiety-like behavior and likely by linking contextual memory to stress.

In chapter 2, we found increased c-Fos expression, an index of neuronal activity, in the dDG and increased anxiety-like behavior following CRF infusion. These results fit with the data described above indicating that increases in HIP activity contribute to anxiety-

like behavior. A closer inspection of the pattern of c-Fos activation revealed that CRF-induced increases appeared to be localized to cells of the hilus (Fig 2.7) which includes inhibitory interneurons and glutamatergic mossy cells (Amaral, 1978; Seress and Ribak, 1983); however, despite efforts, the nature of the cell population activated by CRF (whether GABAergic or glutamatergic) in the present work is not known. Considering that mossy cells are the predominant cell type in this region and are highly excitable (Buckmaster and Schwartzkroin, 1994; Ratzliff et al., 2002) and also that the hilus is particularly vulnerable to cell death following stress (Lowenstein et al., 1992; Kotti et al., 1996; Toth et al., 1997), some effects of stress may be mediated by CRF-induced excitotoxicity in mossy cells, albeit the exact consequences of mossy cell loss are unknown. JDTic treated mice had reductions in CRF-induced increases in c-Fos expression in another population of neurons in the dDG—the excitatory granule cells (Fig 2.7). These changes may reflect a mechanism whereby decreased HIP activation results in reduced anxiety-like behavior and may have important consequences in paradigms of contextual-conditioning—such as footshock-potentiated startle—in which reductions in HIP activity are predicted to affect context-related memory formation or retrieval (Holt and Maren, 1999; Barrientos et al., 2002; Corcoran et al., 2005), thus reducing future anxiety-like behavior in that context.

How KOR blockade can reduce neuronal activation, as measured by c-Fos expression, is unclear although KOR activation has been shown to increase the excitability of granule cells in the dDG (Neumaier et al., 1988; McDermott and Schrader, 2011). There is also evidence in other brain regions that KOR activation can inhibit GABA release (Hjelmstad and Fields, 2003); thus disruption of KOR activation could block

decreases in GABA release and produce greater inhibition of efferent targets. Indeed, KORs are expressed in GABAergic interneurons of the CA1 subregion of the HIP (Halasy et al., 2000), suggesting the possibility of KOR-induced disinhibition in HIP. Additional work to characterize the cell types modulated by CRF and JDTic as observed in c-Fos studies using electrophysiological and double-labeling techniques is a beginning step to understanding interactions between these two systems in the context of anxiety-like behavior. Further, studies using direct drug injections or conditional KO of KORs from the HIP may clarify whether modulation of HIP circuits is necessary for CRF-induced anxiogenic-like behavior and attenuation of this behavior by JDTic.

3. Differences among paradigm and genotype results

3.1 Differences in c-Fos labeling following CRF and footshock

I used c-Fos immunolabeling as an index of neuronal activation to investigate the circuits underlying KOR antagonist effects on CRF-enhanced and footshock-potentiated startle. CRF induced profound brain activation, as every region analyzed contained significantly greater c-Fos expression in CRF- compared to vehicle-infused mice. These data are in contrast to the effects of footshock, which, in the regions analyzed, only increased c-Fos immunoreactivity in the PVN. The PVN is one of the most stress-responsive brain regions as evidenced by significant increases in Fos signal following various aversive stimuli (Beck and Fibiger, 1995; Duncan et al., 1996; Bhatnagar and Dallman, 1998; Funk et al., 2006). Footshock-related induction of c-Fos expression in the PVN narrowly reached significance in my studies, in contrast to the dramatic increases observed by others. This may be due, at least in part, to a ceiling effect manifested by exceptionally high levels of c-Fos labeling in control (no-shock) mice (see

Discussion in Chapter 3). Baseline increases in c-Fos expression are evident when comparing HIP c-Fos immunoreactivity in mice from my footshock studies to mice from my CRF studies. Although the photomicrographs used for counting were not taken at the same time under identical conditions and thus should not be directly compared for statistical analysis, it is clear that no-shock mice have greater activation in HIP subfields compared to aCSF-infused controls (see Table 2.1 and 3.5). These data further suggest that no-shock control mice may have had higher than expected brain c-Fos expression—possibly due to stress (see Chapter 3 Discussion)—which precluded observations of footshock induced increases. Additionally, mice in the footshock study were placed into the startle chambers for 30 min and then returned to their home cage before perfusion, whereas aCSF mice were returned to their home cage immediately after infusion. Since the HIP is a key substrate for representing spatial memory (O'Keefe and Nadel, 1978), this minor procedural difference might have been sufficient to trigger context-related activation that obscured effects of footshock. In an effort to mitigate this possibility, I habituated mice to the testing chamber across several days, but potential changes to the environment at the time of testing—such as odor or temperature—may nonetheless have contributed to HIP activation (Vanderwolf, 1992; Deshmukh and Bhalla, 2003; Karlsson and Blumberg, 2004). Due to the lack of expected footshock effects throughout the HIP and VTA, it is difficult to draw conclusions from the disparate results of the CRF and footshock c-Fos studies. It is clear that CRF produced an intense activation of c-Fos throughout the brain, suggesting it may be a stronger stressor than the footshock protocol. ICV infusions of CRF can activate cognate receptors throughout the brain and induce anxiogenic-like effects for a prolonged period of time (Liang et al., 1992; Bittencourt and Sawchenko, 2000; Meloni

et al., 2006). Because this CRF infusion-induced anxiety-like state is so persistent, it likely does not precisely mimic more naturalistic aversive stimuli-induced anxiety-like states—such as footshock-potentiation of the ASR—that are likely reduced or terminated when the mice are removed from the context. Nevertheless, my c-Fos expression studies demonstrate robust engagement of brain circuits by CRF in contrast to subtle and circumscribed brain activation by footshock.

3.2 Distinct results from KOR antagonist studies and genetic manipulations

I tested $KOR^{-/-}$ mice expecting to see similar reductions in startle potentiation to those observed in systemic KOR antagonist treated mice (Chapters 2 and 3) because KOR function is lacking throughout the brain in both cases. Surprisingly, the behavior of $KOR^{-/-}$ mice was not significantly different from WT littermates in either CRF-enhanced or footshock-potentiated startle. The dissociation between these pharmacological and genetic studies might be explained by differences between the effects of KOR ablation throughout development and acute KOR blockade during adulthood; in the former case, changes in the developmental course of related systems might compensate for KOR ablation (see Chapter 5 Discussion).

Mice lacking KORs in DA neurons were also not significantly different from controls in CRF-enhanced startle, suggesting that the KOR effects that regulate behavior in this paradigm are expressed outside of the mesocorticolimbic system. In contrast, KOR activation within DA neurons mediates, at least in part, footshock effects on startle, as indicated by a significant reduction of footshock-potentiated startle in DAT-$KOR^{lox/lox}$ mice. This pattern of effects might indicate that distinct regions of KOR activation within

circuits regulate CRF versus footshock effects. An intense aversive stimulus (0.8 mA) overcame the reductions in startle potentiation in DAT-KOR$^{lox/lox}$ mice following lower footshock intensities (0.2 and 0.4 mA), which may indicate that high levels of shock engage additional neural systems and/or alternative coping mechanisms. As discussed earlier, 1.0 µg CRF produces a robust increase in c-Fos activation throughout brain systems involved in stress and anxiety-like behavior, suggesting it is a powerful anxiogenic stimulus. Thus, it is possible that conditional KO mice administered CRF were in a state most comparable to that produced by 0.8 mA footshocks and as a result, did not have reductions in CRF-enhanced startle; lower doses of CRF may reveal reductions in DAT-KOR$^{lox/lox}$ mice compared to controls. On the other hand, systemic KOR antagonism was capable of decreasing footshock effects throughout the test session, suggesting that KORs outside of the DA system can also modulate footshock-induced behavior. Collectively, my pharmacological and genetic studies indicate a role for KORs both within and outside the midbrain DA system in the anxiogenic-like effects of CRF and footshock.

While we chose to focus on the midbrain DA system; it is clear that this is not the only system to underlie KOR affects on anxiety, as stress leads to robust KOR activation in non-DA nuclei including the serotonergic DRN (Land et al., 2008). As one example, activation of KORs in the DRN, which sends serotonin (5HT)-containing projections to forebrain areas critical for modulating fear and anxiety responses (Lowry et al., 2005), reduces 5HT release (Tao and Auerbach, 2002) and modulates stress-induced aversion (Land et al., 2009). Whether the 5HT-system works independently or interplays with other stress-responsive systems including DA is not entirely clear. The aversive effects

of KOR-mediated decreases in 5HT are attributable to projections from the DRN to the NAc, an area also rich in DA. Thus, the anxiolytic-like effects of systemic JDTic in response to stress (Chapters 2 and 3), may rely on both inhibition of decreases in 5HT as well as modulation of DA systems, effects that may convergence within the same substrates. Further, the DRN sends direct projections to the striatum and activation of 5HT receptors in the striatum increases extracellular concentrations of DA (Benloucif and Galloway, 1991; Benloucif et al., 1993), raising the possibility that KOR antagonist effects may reduce DA activity in this region indirectly through modulation of 5HT systems. Additional studies to further characterize the role of KORs in systems other than DA may clarify their role in anxiety-like behavior and the nature of interactions among these modulators.

As an alternate approach to our genetic manipulation that ablated KORs in DA neurons (DAT-KOR$^{lox/lox}$ mice), adult KOR$^{lox/lox}$ mice were infused with a viral vector expressing Cre into the VTA to ablate KORs in this structure. These mice were then tested in footshock-potentiated startle to determine whether they replicated the anxiolytic-like effects observed in DAT-KOR$^{lox/lox}$ mice. Unexpectedly, Cre vector-infused mice were not significantly different from GFP vector-infused controls. There are several explanations as to why this manipulation may not have produced a phenotype (see Chapter 5 Discussion), including the possibility that stress-induced KOR activation in VTA neurons may not underlie the anxiogenic-like effects of footshock. Considering that KORs in all DAT-expressing neurons (not just the VTA) were ablated in our conditional KOs, targeted ablation of KORs in DA neurons outside of the VTA may reproduce the anxiolytic-like effects observed in DAT-KOR$^{lox/lox}$ mice. These regions

include the SN, rostral linear nucleus, and periaqueductal gray (Phillipson, 1979; Hasue and Shammah-Lagnado, 2002) all of which express DAT (Shimada et al., 1992; Fujita et al., 1993) and thus would lack KORs in DAT-KOR$^{lox/lox}$ mice. In some mice, we observed a greater spread of the Cre viral vector that included infection of the SN bilaterally. These mice displayed similar behavior to controls, suggesting that if extra-VTA neurons are responsible for the anxiolytic-like effects it is likely not those in the SN. Regardless, further studies to ablate KORs specifically from the SN, rostral linear nucleus and periaqueductal gray are necessary to clarify the role, if any, of KORs in these regions on stress-induced anxiety-like behavior.

4. Potential mechanisms

4.1 KOR effects outside of the VTA

KOR antagonist effects in CRF-enhanced startle were not replicated in DAT-KOR$^{lox/lox}$ mice, suggesting a role for KORs expressed in other regions and/or non-dopaminergic cells. Indeed, stress produces KOR activation in the AMY and DRN, regions implicated in anxiety-like and aversive behavior (Bruchas et al., 2009; Land et al., 2009; Knoll et al., 2011). The work of other groups has provided an elegant mechanism that explains how KOR activation produces aversive effects. This work implicates KORs expressed on terminals of axon projections from the DRN to the NAc (Land et al., 2009). Stress induces KOR-dependent activation of p38 MAPK in DRN serotonergic neurons, which is necessary and sufficient to induce a negative affective state (Bruchas et al., 2007a; Land et al., 2009; Bruchas et al., 2011). These effects are hypothesized to result from inhibition of serotonergic neurons, considering that KOR activation in DRN slice preparations induces p38 MAPK-dependent activation of G-protein-coupled inwardly

rectifying potassium channels (GIRKs) and presynaptic inhibition of excitatory neurotransmission resulting in decreased serotonergic neuron excitability (Lemos et al., 2012a). KORs are also expressed in other stress responsive brain regions that are highly interconnected with the mesocorticolimbic system including the BNST, HIP and septum; however, the role that KORs within these regions on stress and anxiety-related behavior has not been thoroughly examined. Future studies using the floxed KOR mouse generated for these studies in combination with promoter-driven Cre viral vectors have the potential to elucidate the roles of KORs within particular cell populations and brain regions.

4.2 KOR effects on VTA function

Early investigations into the neural substrates of KOR-induced aversion using CPA identified the VTA as a key area of activation (Bals-Kubik et al., 1993). Aversion was postulated to be the result of KOR-mediated decreases in DA release. Indeed, KOR agonists decrease DA release in VTA cell cultures (Ronken et al., 1993; Dalman and O'Malley, 1999) and directly inhibit DA cell firing through GIRKs in slice (Margolis et al., 2003). In addition to postsynaptically inhibiting DA release through hyperpolarization, KOR activation in the VTA can induce presynaptic inhibition of somatodendritic DA at its release sites (Ford et al., 2007). KORs can also regulate VTA activity through the control of glutamate input (Margolis et al., 2005) demonstrating the broad range of KOR control over DA function. Dynorphin terminals synapse onto both TH-labeled (presumably DA neurons) and unlabeled dendrites as well as terminals and astrocytes in the VTA (Pickel et al., 1993) where KOR activation can produce differential responses. Because several dynorphin-expressing nuclei project to the VTA including

those from the hypothalamus, AMY, CPu, and NAc (Fallon and Leslie, 1986), VTA cells expressing KORs may be involved in integrating information from multiple brain circuits or have unique responses based on input and/or projection target. For example, KOR-mediated inhibition of DA neurons varies as a function of projection target (Ford et al., 2006; Margolis et al., 2006b; Margolis et al., 2008). KORs are also located on the terminals of DA projections from the VTA to the NAc and PFC where they can presynaptically inhibit DA release (Di Chiara and Imperato, 1988; Werling et al., 1988; Spanagel et al., 1992; Carlezon et al., 2006; Grilli et al., 2009). This complexity makes it difficult to clearly delineate the circuits underlying our present findings in mice injected with systemic KOR antagonists (which results in blockade of KORs throughout the brain) and DAT-KOR$^{lox/lox}$ mice (which lack KORs on all DA neurons); however, accumulating evidence suggests that alterations in the function of the PFC in particular may regulate, at least in part, the anxiolytic-like effects observed in the current work.

The PFC integrates information for cognitive processing and is involved in emotional learning and memory (Fuster, 2008). DA plays a role in the regulation of PFC function; it is released into this region in response to stress, an effect thought to produce anxiolytic-like behavior in rodents (Thierry et al., 1976; Abercrombie et al., 1989; Deutch and Roth, 1990; Sorg and Kalivas, 1993; Feenstra et al., 2001). In contrast, DA depletion in the PFC induces anxiogenic-like behavior in the EPM (Espejo, 1997; Fernandez Espejo, 2003). Further support for a protective role of PFC DA is provided from studies of neuropeptide S, which produces anxiolytic-like effects thought to be secondary to increases in PFC DA release (Si et al., 2010). Stress-induced enhancement of DA function in the PFC may result from direct activation of VTA

neurons by CRF, considering that CRF-R1 KO in DA neurons results in increased anxiety-like behavior accompanied by reductions in PFC DA release in response to stress (Refojo et al., 2011). Thus KORs have the ability to modulate anxiogenic-like responses to stress through the control of DA release within the PFC (Margolis et al., 2006b; Tejeda et al., 2010b). Although the current work has more precisely determined the role of KORs in the VTA as compared to drug microinfusions by using Cre/loxP recombination to target DA neurons specifically, the heterogeneity of DA neuron subtypes expressing KORs in the VTA suggests that even more precise targeting of specific DA subpopulations may lead to a better understanding of the particular role of KORs within each component of the mesocorticolimbic system.

4.3 Hypothetical framework to explain behavioral effects

The paradigms of anxiety-like behavior that I used in this thesis (CRF-enhanced startle and footshock-potentiated startle) were chosen to elicit a sustained fear response that more accurately models clinical aspects of anxiety disorders as compared to cued fear conditioning, which elicits phasic fear and relies partly on distinct brain regions (Davis et al., 2010). In models of both sustained and phasic fear, aversive stimuli activate the BLA which sends projections to the CeA and BNST. The medial subregion of the CeA is responsible for rapid phasic fear responses, while activation of the lateral CeA elicits CRF release into the BNST producing a slower onset, sustained fear response (Davis et al., 2010). This model is supported by work indicating that the BNST is critical for CRF- and light-enhanced startle—paradigms that produce sustained levels of fear throughout the test session—but not fear-potentiated startle—a paradigm in which startle is facilitated only following a short (3.7 sec) cue (Hitchcock and Davis, 1986; Hitchcock

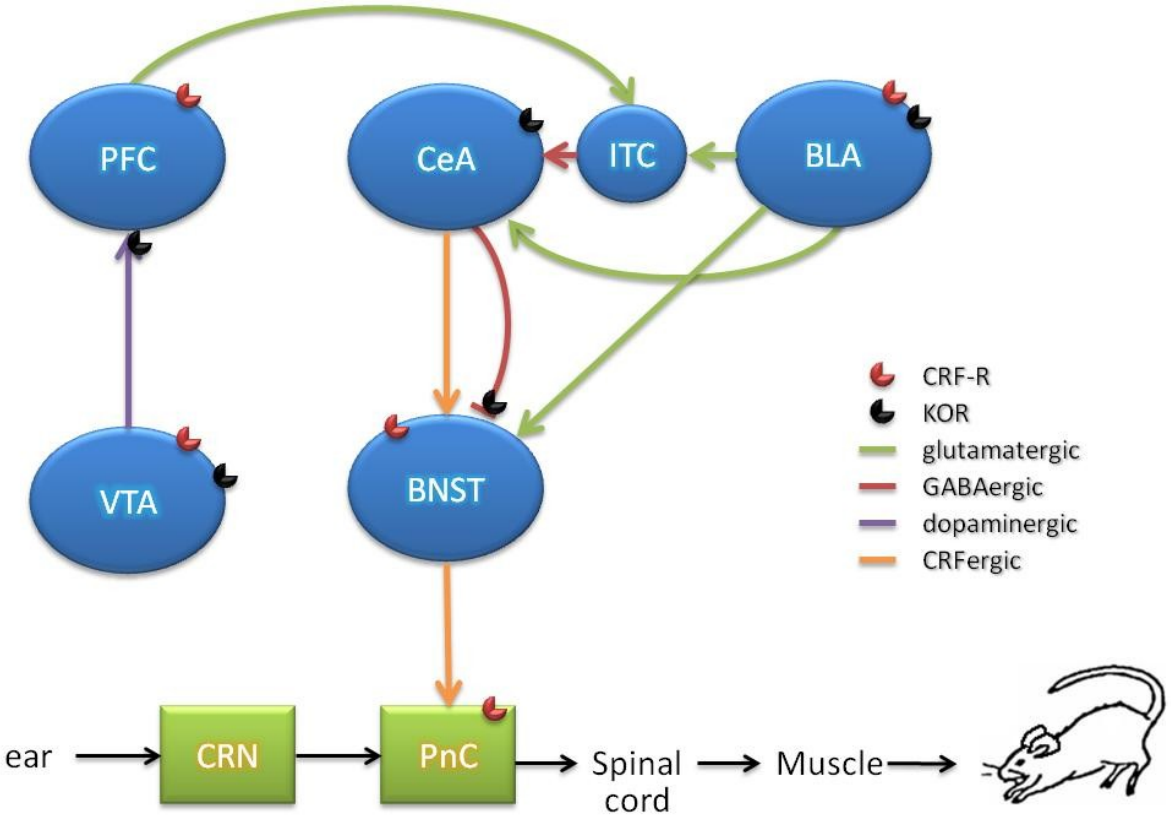
and Davis, 1991; Lee and Davis, 1997; Walker and Davis, 1997). Thus BNST activation is a key component for increases in startle reactivity in the CRF-enhanced and footshock-potentiated startle paradigms used in my dissertation.

A highly simplified circuit hypothesized to underlie KOR effects on anxiety is illustrated in **Fig. 6.1** (adapted from Davis (2010) and Meloni (2006)). According to this model, sensory activation of the BLA by aversive stimuli would increase activity in the CeA through direct projections to this region. The CeA sends abundant projections to the BNST that provide the primary source of CRF to this region (Sakanaka et al., 1986; Sun et al., 1991; Petrovich and Swanson, 1997; Dong et al., 2001a). Stress would cause the release of CRF and subsequent activation of the BNST, a region rich in CRF-Rs (Wynn et al., 1984). CRF and footshock-induced increases in startle would result, in part, from direct projections from the BNST to the PnC, a critical relay site in the startle pathway from ear to muscle (Davis et al., 1982; Rosen et al., 1991). This response may be modulated by PFC effects on intercalated cell (ITC) masses—GABAergic neurons located between the BLA and CeA that inhibit AMY output (Millhouse, 1986; Pare et al., 2004). Direct projections from the PFC to the ITCs have been demonstrated in primates and rodents (McDonald et al., 1996; Chiba et al., 2001; Ghashghaei and Barbas, 2002), and regulate AMY output (Maren and Quirk, 2004; Pare et al., 2004). Indeed, stimulation of the PFC increases c-Fos labeling in the ITCs (Berretta et al., 2005) and reduces the response of CeA output neurons to BLA activation (Quirk et al., 2003).

In times of stress, PFC activation might occur following mesocortical DA release (Thierry et al., 1976; Roth et al., 1988) under the control of presynaptic KORs. Release of dynorphin and activation of KORs may be especially prominent in response to repeated or prolonged stress, which would reduce DA release and subsequent PFC activity and lead to increases in anxiety-like behavior (e.g. enhanced startle). Indeed, DA activity in the PFC changes with repeated stress (Tanaka et al., 2012). Whereas acute defeat activates the mesocortical DA pathway, 10 days of defeat leads to an attenuation of DA turnover that is accompanied by increases in social avoidance and anxiogenic-like behavior (Tanaka et al., 2012). Because KOR-induced decreases in DA release within the PFC (Margolis et al., 2006b) would presumably be absent in DAT-$KOR^{lox/lox}$ mice, the anxiolytic-like effects observed in footshock-potentiated startle in these mutants may result from DA-induced PFC activation of ITCs, the postulated "off" switch of the AMY (Quirk and Mueller, 2008).

Figure 6.1 Model illustrating the hypothetical involvement of mesocortical projections in stress-induced facilitation of the ASR. Sensory stimuli associated with aversive events activate the BLA, which promotes CRF release in the BNST via the CeA, and activates the BNST directly. In turn, the ASR is enhanced through direct BNST to PnC projections. CeA output is inhibited by PFC-induced ITC activation, reducing BNST modulation of the acoustic startle pathway. As ablation of KORs in the VTA attenuates the increases in startle produced by footshock, I hypothesize that disruption of mesocortical KOR function enhances DA release and PFC activation. KOR and CRF effects outside of the VTA (e.g. in the AMY, BNST and PnC) may also modulate this circuit and account for the differences observed in my pharmacological and genetic studies. AMY, amygdala; ASR, acoustic startle reflex; BLA, basolateral nucleus of the amygdala; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; CRF, corticotropin-releasing factor; CRN, cochlear root neurons; DA, dopamine; ITC, intercalated cell masses; KOR, kappa-opioid receptor; PFC, prefrontal cortex; PnC, caudal nucleus of the pontine reticular formation; VTA, ventral tegmental area

Figure 6.1 (Continued) Model illustrating the hypothetical involvement of mesocortical projections in stress-induced facilitation of the ASR



This hypothesized circuit may help clarify the results in KOR antagonist versus KOR-deficient mice in CRF-enhanced and footshock-potentiated startle. ICV CRF infusions like those used for my CRF-enhanced startle studies would be capable of directly activating CRF-Rs of the CeA, BNST and PnC (Wynn et al., 1984; Smagin et al., 2001), thus bypassing the anxiolytic-like effects of KOR ablation in DA neurons. According to this putative scheme, CRF-enhanced startle would be equivalent in DAT-KOR$^{lox/lox}$ mice and controls, as was observed. Likewise, additional KOR antagonist effects outside of the mesocortical system would contribute to attenuation of startle as observed in my JDTic studies (Chapters 2 and 3). As one example, amygdalar KORs regulate anxiety-like behavior and are present on GABAergic terminal inputs to the BNST, where they inhibit GABA release that would tend to dampen BNST activation (Li et al., 2012). Thus systemic blockade of KORs can have effects at multiple levels of the proposed circuit to reduce BNST activation and anxiogenic-like effects of stress on the ASR.

It is important to note that in the proposed model of stress effects on the ASR, we would expect to see increased activation of VTA neurons that project to the PFC in JDTic-treated mice; however, c-Fos expression studies (Chapter 2) indicate significant reductions. However, there is emerging evidence that DA cells in the VTA have disparate projections that can respond to inputs independently. In fact, some reports indicate an increase in VTA firing following social defeat stress in susceptible mice that confers vulnerability through actions that occur within the NAc (Krishnan et al., 2008; Cao et al., 2010). As such, decreases in activation of this particular VTA projection would be beneficial (anxiolytic). In the current work, I was unable to identify PFC-projecting DA neurons specifically and therefore could not determine whether

mesocortical and mesolimbic DA neurons deviated in their response to JDTic as would be predict by this model. c-Fos expression experiments in which VTA neurons are retrogradely labeled by projection target may help to clarify whether DA neurons projecting to the PFC are activated in response to stress.

5. Conclusions and future directions

The KOR system is implicated in anxiety-like behaviors particularly in response to stress. While KOR antagonists produce anxiolytic-like effects on their own and block the effects of stress, KOR agonists mimic stress to induce depressive-and anxiogenic-like behavior. Previous work has shown that these effects rely, at least in part, on KOR activation within the AMY and serotonergic neurons of the DRN. The work presented here offers the first data to demonstrate a role for KORs on DA neurons in stress-induced anxiety-like behavior. Together, these data suggest that the KOR system has broad control over neurotransmitter systems throughout the brain to regulate circuits involved in responses to stress. While normal KOR function is important to antagonize other brain systems and maintain response adaptability (Pan, 1998), dysfunction in this system may be responsible for psychiatric disorders such as depression and anxiety.

Here I propose that KOR control of DA release into the PFC may partly underlie the anxiolytic-like effects of KOR blockade. To support this model, it will first be important to determine whether DA release is enhanced in DAT-KOR$^{lox/lox}$ mice through microdialysis studies. Further work using optogenetic techniques may clarify the role of the mesocortical KOR system in anxiety-like behavior through specific stimulation of these projections following KOR excision or rescue. Such experiments can be achieved

through stimulation of the PFC after VTA expression of channelrhodopsin (Ch2) under the control of a DAT promoter in our DAT-KOR$^{lox/lox}$ mice. This would enable specific control of KOR-lacking DA projections from the VTA to PFC and would hypothetically reduce anxiety-like behavior. Previously, optogenetic stimulation of the PFC has been shown to reduce some measures of anxiety-like behavior in mice (Covington et al., 2010) providing some evidence that DA-induced activation of this area may produce similar results. Such studies may lead us closer to the role of KORs within the complex circuitry underlying anxiety disorders.

Appendix

Corticotropin-releasing factor (CRF)-induced disruption of attention in rats is blocked by the kappa-opioid receptor antagonist JDTic

Ashlee Van't Veer[1], Jessica M. Yano[1], F. Ivy Carroll[2], Bruce M. Cohen[1], and William A. Carlezon Jr.[1]

[1]Department of Psychiatry, Harvard Medical School, McLean Hospital, Belmont, MA 02478, and [2]Center for Organic and Medicinal Chemistry, Research Triangle Institute, Research Triangle Park, NC 27709

Author contributions: AVV and WAC designed the experiments. AVV administered drugs and performed the tail flick. AVV and JMY conducted 5-choice serial reaction time task tests. FIC synthesized and provided JDTic. WAC and AVV analyzed data and wrote the paper. All authors read and approved the final manuscript.

Abstract

Stress often disrupts behavior and can lead to psychiatric illness. Considerable evidence suggests that corticotropin-releasing factor (CRF) plays an important role in regulating the effects of stress. CRF administration produces stress-like effects in humans and laboratory animals, and CRF levels are elevated in individuals with stress-related illness. Recent work indicates that kappa-opioid receptor (KOR) antagonists can block CRF effects, raising the possibility that at least some of the effects of stress are mediated via KORs. Here we examined the effects of CRF on performance in the 5-choice serial reaction time task (5CSRTT), a test used to quantify attention in rodents, as well as functional interactions between CRF and KORs. Male Sprague-Dawley rats were trained in the 5CSRTT and then each was implanted with an intracerebroventricular (ICV) cannula. After recovery and re-stabilization of performance, they received a single intraperitoneal (IP) injection of vehicle or JDTic (10 mg/kg), a KOR antagonist with long-lasting (>14 days) effects. In subsequent sessions, rats received ICV infusions of CRF (0.25-1.0 μg) or vehicle and were tested 60 min later. CRF dose-dependently disrupted performance as reflected by decreases in correct responding, increases in omission errors, increases in latencies to respond correctly, and increases in time to complete the session. JDTic attenuated each of these CRF-induced deficits while having no effects on its own. The persistent ability of JDTic to disrupt KOR function was confirmed using the tail immersion assay. These findings indicate that KOR antagonists can prevent acute stress-related effects that degrade performance in tasks requiring attention.

Introduction

Stress can have disruptive effects on behavior, cognition, and motivation (Knoll and Carlezon, 2010; Campeau et al., 2011). Exposure to severe or repeated stress can cause psychiatric illnesses including anxiety and depressive disorders such as PTSD (Keane et al., 2006; Keller et al., 2007; Kessler et al., 2010). Stress-related illnesses are debilitating and burdensome because they tend to be persistent, resistant to treatment, and co-morbid with substance abuse disorders (Chilcoat and Breslau, 1998; Greenberg et al., 1999; Koob and Kreek, 2007). Currently there are no treatments available that reliably block the effects of stress or have broad efficacy in reversing the long-term effects of prior stress exposure.

There is considerable evidence that corticotropin-releasing factor (CRF) plays an important role in regulating stress effects. CRF is a neuropeptide that is released in the brain in response to stress (Koob, 1999). Administration of CRF produces many of the same physiological and behavioral effects as stress in people and laboratory animals (Hauger et al., 2009), and people with stress-related psychiatric illness have higher levels of CRF in cerebrospinal fluid (CSF) and blood (Bremner et al., 1997a; Sautter et al., 2003; de Kloet et al., 2008). Although much is known about the neural mechanisms by which CRF regulates stress (Bangasser and Valentino, 2012), it has remained difficult to develop clinically effective anti-stress agents that act directly at CRF receptors (Zorrilla and Koob, 2010).

Accumulating evidence suggests that important aspects of the stress-related effects of CRF are mediated by kappa-opioid receptors (KORs) (Bruchas and Chavkin, 2010;

Knoll and Carlezon, 2010), the receptor at which the endogenous opioid dynorphin acts (Chavkin et al., 1982). For example, the prototypical KOR antagonist nor-binaltorphimine (norBNI) blocks CRF-induced dysphoria in the place conditioning test (Land et al., 2008) and reductions in open arm time in the elevated plus maze (Bruchas et al., 2009). Our group has shown in preliminary tests that JDTic, another highly selective KOR antagonist that is structurally unrelated to norBNI (Carroll et al., 2004), also blocks CRF-induced elevations in acoustic startle behavior (Van't Veer et al., 2011). The observations that CRF-induced phosphorylation of KORs is blocked by KOR antagonists (Land et al., 2008) and CRF-induced anxiety behavior is reduced in dynorphin knockout mice (Bruchas et al., 2009) provide molecular evidence for links between CRF and KOR systems. Interactions between these systems have been thoroughly characterized within the raphe nucleus (Bruchas et al., 2011) but may also occur in other brain regions (Pliakas et al., 2001; Newton et al., 2002; Shirayama et al., 2004; Knoll et al., 2011; Muschamp et al., 2011b). The notion that KOR antagonists block the effects of stress fits well with other evidence that these agents have antidepressant-like (Pliakas et al., 2001; Newton et al., 2002; Mague et al., 2003; Shirayama et al., 2004) and anxiolytic-like effects including the ability to block acquisition of fear-potentiated startle (Knoll et al., 2007; Knoll et al., 2011), a procedure often used to study PTSD (Mahan and Ressler, 2012). In addition, KOR agonists can produce key behavioral signs of stress (Mague et al., 2003; McLaughlin et al., 2003a; Todtenkopf et al., 2004; McLaughlin et al., 2006b). When considered together, these findings raise the possibility that pretreatment with KOR antagonists could reduce or prevent effects of stress, representing an alternative approach to modulating the behavior-disrupting effects of CRF.

The present studies were designed to examine how CRF affects a subset of cognitive behaviors in rodents, and whether pretreatment with a KOR antagonist (JDTic) mitigates any stress-like effects. Cognitive behavior was quantified using the 5-choice serial reaction time task (5CSRTT), a food-motivated test that is analogous to the continuous performance task used to study attention in humans (Rosvold et al., 1956; Robbins, 2002). The 5CSRTT yields metrics that quantify attention, reaction time, motivation, and impulsivity (Robbins, 2002; Paine et al. 2007; Nemeth et al., 2010). Stress is known to degrade performance in tasks requiring attention or concentration in humans (Campeau et al., 2011), and poor concentration is one of the diagnostic criteria for stress-related psychiatric illnesses such as PTSD (American Psychiatric Association, 2000). Previous work has demonstrated that JDTic produces long-lasting (>14 days) disruptions of KOR function (Carroll et al., 2004) and that the behavioral effects of JDTic and norBNI are virtually identical (Knoll et al., 2007; Knoll and Carlezon, 2010). To confirm that a single injection of JDTic produced disruption of KOR function for the duration of our tests in the 5CSRTT, we examined the ability of the KOR agonist U50,488 to produce antinociceptive effects in the tail immersion assay (Smith and French, 2002).

Materials and Methods

Rats: A total of 14 male Sprague-Dawley rats (Charles River, Raleigh, NC; 250–275 g at the start of the experiment) were used. Rats were housed two per cage upon arrival and kept on a 12-hour/12-hour light-dark cycle (lights on at 7:00 AM) and given 1 week to acclimate with free access to food (Purina Rat Chow; Ralston Purina, St. Louis, MO) and water. Beginning 2 days before the start of training, the rats were food restricted to

85% of their free-feeding weight. Experiments were conducted in accordance with the National Institutes of Health, and McLean Hospital guidelines for the care and use of laboratory animals.

5CSRTT: The apparatus and training have been described previously (Paine et. al., 2007). Briefly, the operant chambers (Med-Associates, St. Albans, Vermont) were contained within sound-attenuating cubicles. One wall contained five apertures capable of LED illumination and outfitted with infrared detectors to record nose-pokes. The opposite wall contained a food reward receptacle also capable of illumination and nose-poke detection that was connected to a pellet dispenser. Rats were handled for 3 days before the start of training. During the next 3 days, rats were trained to retrieve food pellets (45 mg; Bio-Serv, Frenchtown, NJ) from the food magazine. Rats were then trained to nose-poke in one of five spatial locations within 5 sec of the presentation of a brief stimulus light (0.5 sec). A timely response in this aperture resulted in delivery of 1 food pellet. Incorrect nose-pokes in the other apertures resulted in a 5-sec time-out. Similarly, failing to respond (omission) or responding during the 5-sec inter-trial interval (premature response) resulted in a 5-sec time-out. Sessions were 90 trials or 30 min, whichever came first. Performance measures of primary interest were: % correct ((correct responses/[correct + incorrect+ omitted responses])*100), accuracy ((correct responses/[correct + incorrect responses])*100), % omissions ([total omissions/number of trials]*100; trials in which no response was emitted), latency to make a correct response (the time from the stimulus onset to a correct response; a putative indicator of speed of processing or decision-making), reward latency (the time from a correct response to the collection of the food pellet; a putative indicator of motivation),

premature responses (responses during the ITI; a putative indicator of impulsivity), and time to complete the task (a putative indicator of overall performance capabilities). The criteria to advance to the next stages of the experiments were >60% correct responses and <20% omissions for 5 consecutive days.

Stereotaxic Surgery: Upon meeting performance criteria, rats underwent surgery to implant an intracerebroventricular (ICV) cannula. Each rat was anesthetized with an intraperitoneal (IP) injection of pentobarbital (65 mg/kg) supplemented with subcutaneous atropine (0.25 mg/kg) to minimize bronchial secretions, and placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA). For each rat, a stainless steel guide cannula (23-gauge, Plastics One, Roanoke, VA) with a dummy stylet extending 1.5 mm beyond the tip was lowered into the right lateral ventricle at coordinates relative to bregma; anteroposterior = -0.8 mm, mediolateral = 1.3 mm, and lowered -3.5 mm ventral to dura. Dental acrylic (Stoelting, Wood Dale, IL) secured the cannula to screws (Plastics One, Roanoke, VA) attached to the skull. Rats were housed individually after surgery to recover for 5-7 days, and then tested until their performance had re-stabilized to baseline levels (±10%) while also fulfilling the basic response criteria (>60% correct responding, <20% omissions). Microinfusions were performed by removing the dummy stylet and replacing it with a 30-gauge infusion stylet (Plastics One) attached to a Hamilton microsyringe (10 µl) by polyethylene tubing. ICV infusions of CRF (0.25, 0.5 or 1.0 µg) or vehicle (artificial cerebrospinal fluid [aCSF]; Harvard Apparatus, Holliston, MA) were performed over a 2-min period at a rate of 0.5 µL/min, with an additional 2 minutes of diffusion time before the stylet was removed and the dummy stylet was replaced. Testing began 60 min after infusion.

5CRSTT Studies: Drugs and Design. CRF was purchased from American Peptide (Sunnyvale, CA) and dissolved in aCSF. JDTic was synthesized at Research Triangle Institute (Research Triangle Park, NC) and dissolved in 0.9% saline; 10 mg/kg (based on the salt form of the drug) was selected because this dose produces strong anxiolytic-like effects in rats (Knoll et al., 2007). Rats first received an infusion of aCSF to ensure the infusion procedure did not affect performance, and to obtain data to serve as baseline. Forty-eight hr later, the rats received an injection of either JDTic (10 mg/kg, IP; n=7) or vehicle (1.0 mL/kg, IP, n=7). A 24-hr pretreatment period was used before beginning behavioral testing to optimize KOR selectivity (Carroll et al., 2004; Knoll et al., 2007). Rats were subsequently tested with CRF in the following order: 0 (aCSF), 0.5, 1.0 and 0.25 µg. Rats did not receive subsequent treatments until their performance had re-stabilized to baseline levels (±10%) while also fulfilling the basic response criteria (>60% correct responding, <20% omissions).

Tail Immersion Assay: Drugs and Design. After the final test in the 5CSRTT, the ability of a single injection of JDTic to produce long-lasting disruptions of KOR function was assessed by quantifying KOR agonist-induced analgesia in the tail immersion assay (Smith and French, 2002). A stopwatch was used to measure the latency at which each rat removed its tail from a 52°C (±1°C) water bath. A baseline measurement was obtained before treatment with the KOR agonist (±)-*trans*-U50,488 methanesulfonate (15 mg/kg, IP, dissolved in 0.9% saline; dose based on the salt form of the drug). Latencies were re-assessed 60 min after KOR agonist treatment. A cutoff time of 15 sec was used to prevent tissue damage to the tail.

Histological analysis: After the tail immersion assay, rats were overdosed with pentobarbital (130 mg/kg, IP) and perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were kept overnight in 30% glycerol before sectioning (40 µm). ICV cannula placements were verified in histological analyses by an observer unaware of the treatment conditions. Data from rats in which the tip of the cannula was found to be embedded in brain tissue adjacent to the lateral ventricle, rather than being located within the lateral ventricle itself, were excluded from the statistical analyses since this could affect the quality of the ICV infusion.

Statistics: For the 5CSRTT, 2-way (treatment x session) analyses of variance (ANOVAs) with repeated measures were used to compare the effects of an ICV infusion alone (baseline) with the effects of the ICV infusion plus the pretreatment (saline or JDTic). Separate 2-way (pretreatment x treatment) ANOVAs with repeated measures were used to compare the effects of various doses of CRF in saline- or JDTic-treated mice. Analyses were performed for each individual metric. For the tail immersion assay, a 2-way (pretreatment x treatment) ANOVA with repeated measures was used to examine the effects of prior treatment with JDTic on U50,488-induced antinociception, and a t-test was used to examine group differences in the timing of the tail immersion assay. Significant interactions in the ANOVAs were further analyzed using Newman-Keuls *post hoc* tests, whereas significant main effects in the absence of interactions were further analyzed using Simple Main Effects tests.

Results

Three rats (1 vehicle, 2 JDTic) were excluded because histological analyses revealed that the tips of their guide cannula had been embedded in tissue adjacent to the lateral ventricle. The tips of the ICV cannulas for the remaining 6 vehicle-treated rats and 5 JDTic-treated rats were located entirely within the lateral ventricle (**Fig. A.1**) and thus data from these rats were included in the final statistical analyses.

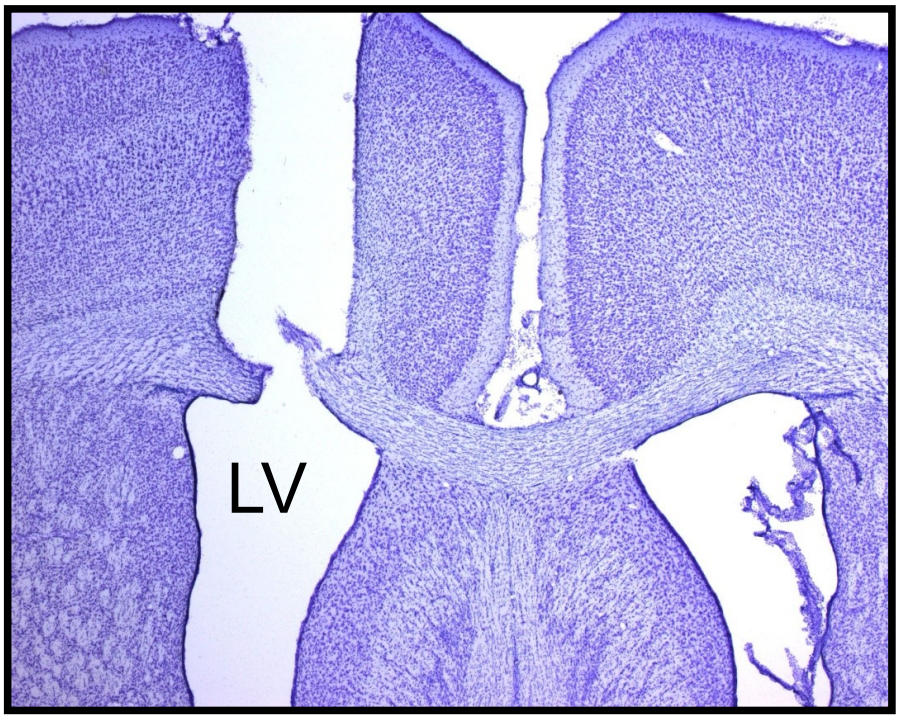


Figure A.1 Representative micrograph of ICV cannula tract in cresyl violet-stained tissue. Rats were excluded if the tip of the cannula was embedded in the brain tissue surrounding the lateral ventricle (LV).

CRF produced JDTic-sensitive disruptions in performance in 4 of the 5CSRTT metrics: percent correct responding, percent omission errors, latency to make a correct response, and time to complete the task (**Fig. A.2**). For correct responding, administration of JDTic did not produce any effects on its own (**Fig. A.2A, left panel**). However, the effects of CRF depended on an interaction of pretreatment (saline or JDTic) and treatment (CRF dose) ($F[3,27]=3.60$, $P<0.05$) (**Fig. A.2A, right panel**). *Post hoc* analyses of within-subject effects revealed that CRF significantly reduced the percentage of correct responding at 0.5 µg and 1.0 µg doses (P's<0.01) in saline-treated rats, but only at the 1.0 µg dose ($P<0.01$) in JDTic-treated rats. Between-group analyses revealed that correct responding was significantly higher in JDTic-treated rats than in saline-treated rats at the 0.5 µg dose of CRF ($P<0.01$). Likewise, for omission errors, JDTic did not produce any effects on its own (**Fig. A.2B, left panel**), but the effects of CRF depended on a pretreatment x treatment interaction ($F[3,27]=3.33$, $P<0.05$) (**Fig. A.2B, right panel**). *Post hoc* analyses of within-subject effects revealed that CRF significantly increased the percentage of omitted responses at 0.5 µg and 1.0 µg doses (P's<0.01) in saline-treated rats, but only at the 1.0 µg dose ($P<0.01$) in JDTic-treated rats. Between-group analyses revealed that the percentage of omitted responses was lower in JDTic-treated rats than in saline-treated rats at the 0.5 µg ($P<0.01$) and 1.0 µg ($P<0.05$) doses of CRF. While JDTic on its own did not have any effects on latency to make correct responses (**Fig. A.2C, left panel**), the effects of CRF depended on main effects of pretreatment ($F[1,9]=5.82$, $P<0.05$) and treatment ($F[3,27]=9.48$, $P<0.01$) (**Fig. A.2C, right panel**). *Post hoc* analyses of between-group effects using Simple Main Effects tests revealed that the latencies to respond correctly were lower in JDTic-treated rats than in saline-treated rats at the 0.5 µg dose of CRF

(F[1,9]=10.9, *P*<0.01). JDTic on its own also did not have any effects on time to complete the task (i.e., to finish the test session) (**Fig. A.2D, left panel**), but the effects of CRF depended on a pretreatment x treatment interaction (F[3,27]=3.00, *P*<0.05) (**Fig. A.2D, right panel**). *Post hoc* analyses of within-subject effects revealed that CRF significantly increased the time to complete the task at the 0.5 μg (*P*<0.05) and 1.0 μg doses (*P*<0.01) in saline-treated rats, but not at any of the doses tested in the JDTic-treated rats. Between-group analyses revealed that time to complete the task was shorter in JDTic-treated rats than in saline-treated rats at the 0.5 μg dose of CRF (*P*<0.01).

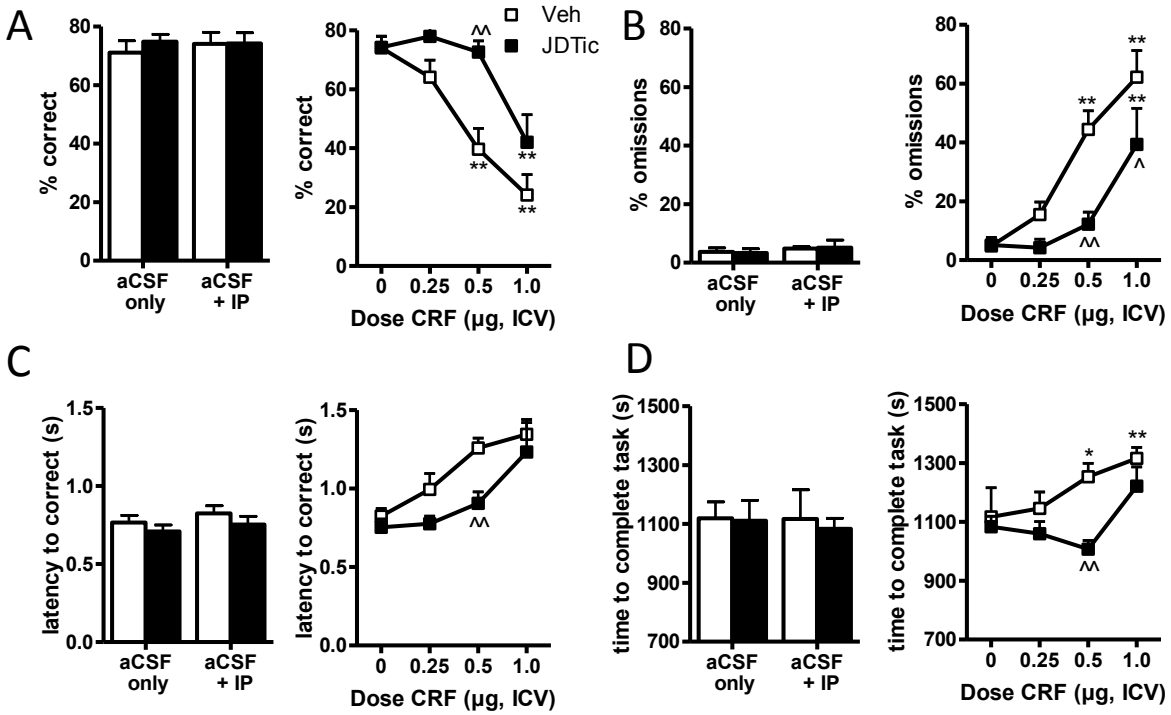


Figure A.2 Effects of JDTic pretreatment on the ability of CRF to affect performance in the 5CSRTT. Left panel represents effects of pretreatment alone on baseline ***A.*** Percent Correct Responses, ***B.*** Percent Omissions, ***C.*** Latency to Correct Responses (in sec), and ***D.*** Latency to Complete the Task (in sec); right panel represents effects after various doses of CRF. **P*<0.05, ***P*<0.01 within group comparisons, ^*P*<0.05, ^^*P*<0.01 between group comparisons, Newman-Keuls *post hoc* t-tests.

In addition (**Table A.1**), CRF produced effects on other metrics that were not affected by JDTic treatment: there were main effects on accuracy ($F[3,27]=3.56$, $P<0.05$), latencies to collect the reward (food pellet) ($F[3,27]=5.87$, $P<0.01$), and number of premature responses ($F[3,27]=3.33$, $P<0.05$).

In the tail immersion assay, latencies to remove the tail from the hot water depended upon a significant pretreatment x treatment interaction ($F[1,9]=9.34$, $P<0.05$) (**Fig. A.3**). *Post hoc* analyses of within-subject effects revealed that latencies were significantly higher 60 min after U50,488 (15 mg/kg, IP) in the saline-treated group ($P<0.01$) but not in the JDTic-treated group. Between-group analyses revealed no group differences at baseline but significantly higher latencies in the saline-treated rats 60 min after U50,488 treatment ($P<0.01$). Rats in the JDTic-treated group received the tail immersion test 11.0 (±1.6) days after pretreatment whereas rats in the saline-treated group received it 15.8 (±6.4) days after pretreatment. This difference was not statistically significant ($t[9]=1.76$, not significant); much of the variability in the saline-treated group was attributable to a rat that was resistant to re-stabilization after treatment with the 1.0 µg dose of CRF.

Table A.1 Additional 5CSRTT Metrics

Metric	Group	Pretreat	aCSF	CRF 0.25	CRF 0.5	CRF 1.0	Statistics[a]
Accuracy	Vehicle	74.0 ± 4.6	77.7 ± 4.2	75.8 ± 4.4	69.0 ± 6.1	58.2 ± 6.6	F[3,27]=3.56
	JDTic	76.2 ± 2.4	78.2 ± 4.3	81.6 ± 4.9	82.8 ± 2.3	67.8 ± 2.8	*P*<0.05
Reward Latency	Vehicle	1.53 ± 0.10	1.65 ± 0.14	1.93 ± 0.33	2.09 ± 0.41	2.35 ± 0.36	F[3,27]=5.87
	JDTic	1.52 ± 0.15	1.48 ± 0.11	1.59 ± 0.16	1.63 ± 0.17	2.40 ± 0.30	*P*<0.01
Premature	Vehicle	21.8 ± 5.8	20.8 ± 10.2	15.4 ± 4.1	10.8 ± 1.6	9.7 ± 1.8	F[3,27]=3.33
	JDTic	21.8 ± 6.8	17.2 ± 2.9	16.4 ± 3.7	4.4 ± 2.0	7.6 ± 1.7	*P*<0.05

[a]Main effects of Treatment (CRF Dose). No Main effects of Pretreatment or Interactions

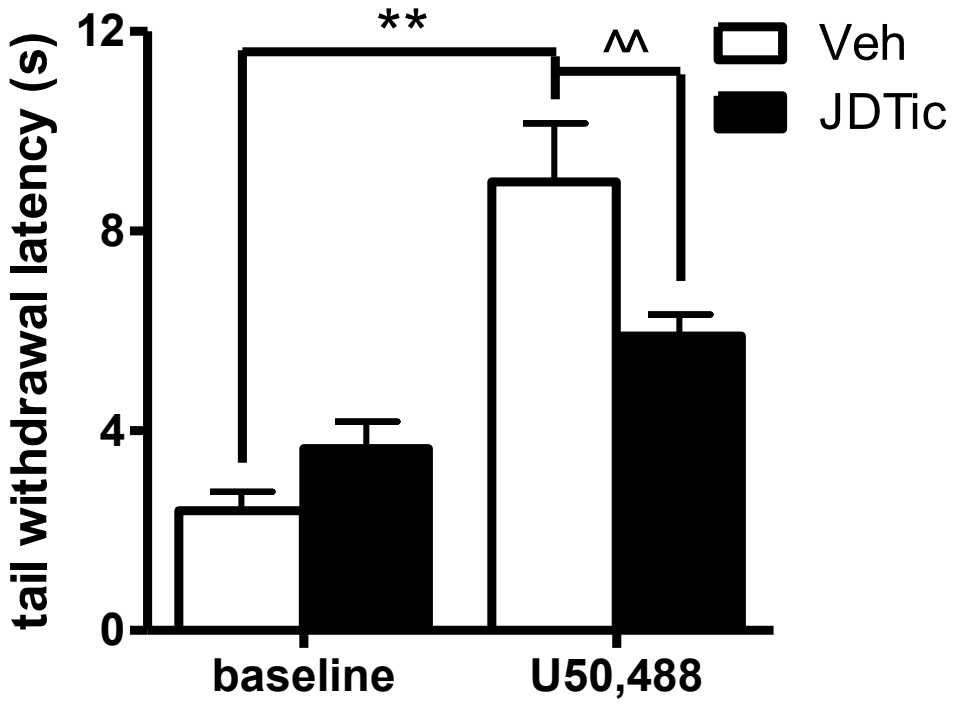


Figure A.3 Effects of JDTic pretreatment on latency to withdraw the tail (in sec) in the tail immersion assay at baseline or 60 min after administration of the KOR-selective agonist U50,488 (15 mg/kg, IP). ***P*<0.01 within group comparisons, ^^*P*<0.01 between group comparisons, Newman-Keuls *post hoc* t-tests.

Discussion

We report 3 important findings. First, we show that administration of CRF, an approach known to mimic effects of stress in humans and laboratory animals (Bangasser and Valentino, 2012), can produce dramatic disruptions of performance in rats as measured in the 5CSRTT. This finding is novel, considering that previous work suggests that CRF can enhance performance in the 5CSRTT at lower doses (0.1 μg) and shorter pretreatment times (20 min) (Ohmura et al., 2009), and suggests an inverted U-shaped function of CRF on cognitive behavior. Second, we show that pretreatment with a single injection of JDTic, a highly selective KOR antagonist with long-lasting effects (Carroll et al., 2004), can reduce or prevent numerous acute stress-related effects that degrade performance in tasks requiring attention. Finally, we confirm that the behavioral effects of JDTic are persistent in rats, producing a virtually complete blockade of KOR function for at least 11 days after administration. These findings are broadly consistent with previous work indicating that KOR antagonists can block the effects of stress (Bruchas et al., 2010; Knoll et al., 2010), but extend it to a more complex aspect of cognitive behavior (attention/concentration) using a procedure that is directly analogous to that used to measure attention in humans (Robbins, 2002).

The primary indicator that CRF disrupted performance in these studies is decreases in the percentage of correct responses. CRF also increased the percentage of trials in which the rats failed to respond (omission errors) and increased the latency to make a correct response, an effect that may reflect reduced speed of processing or decision-making (Robbins, 2002; Paine et al., 2007; Nemeth et al., 2010). The fact that CRF increased the time required to complete the task (i.e., collect 90 rewards before the end

of the 30-min test session) likely reflects the accumulation of 5-sec time out periods after omitted responses and the small but significant increases in decision-making time over the course of the session. Although some of these 5CSRTT metrics are clearly related (e.g., latencies to respond and time to complete the task), previous work demonstrates that key metrics can vary independently and that different drug classes can produce different patterns of alterations in responding (Paine et al., 2007; Nemeth et al., 2010). Pretreatment with JDTic attenuated each of these CRF effects. JDTic was most effective at intermediate doses of CRF that caused significant disruptions of behavior, but its effects were less evident at high doses of CRF that caused more profound stress-like behavior, suggesting lower efficacy in preventing the acute effects of extreme amounts or degrees of stress. Importantly, JDTic did not have any effects of its own on any of these measures. While some of the JDTic effects might be suggestive of behavioral activation, stimulant effects have not been observed with this class of drugs. For example, we did not observe any effects of KOR antagonists on locomotor activity at doses that produce antidepressant-like or anxiolytic-like effects (Mague et al., 2003; Knoll et al., 2007), or alterations in reward-driven behavior or response capabilities at doses that block the prodepressive-like effects of KOR agonists on motivation (Todtenkopf et al., 2004). JDTic also blocks (rather than primes) stress-induced reinstatement of cocaine self-administration in rats (Beardsley et al., 2005), another indicator of a lack of effects that would raise concerns about stimulant effects or abuse potential of this class of drugs. The fact that JDTic did not attenuate the effects of CRF on accuracy, time to collect the reward, or premature responses reinforces the notion that individual 5CSRTT metrics are not inextricably linked to one another. The CRF-induced reductions in accuracy reflect gradual increases in “commission errors”,

where the rat responds but at the incorrect aperture. JDTic caused nominal reductions in the accuracy-disrupting effects of CRF, but these effects were not statistically significant. Overall, this pattern of effects (increases in omission errors but not commission errors) resembles that seen in this test following administration of ketamine (Nemeth et al., 2010), a drug known to disrupt attention in humans (Knott et al., 2011). Both omission and commission errors can have significant adverse consequences during times of stress.

The mechanisms by which KOR antagonists prevent the effects of stress in general or of CRF specifically are not fully understood. There is no evidence from published studies that KOR antagonists bind to CRF receptors. While it would be speculative to attribute our effects to actions in any particular brain area or circuit, there are some obvious candidates. As one example, there is evidence that KOR antagonists produce anti-stress effects via interactions with the intracellular signaling molecule p38α MAPK (Bruchas et al., 2011). Stress produces increases in the activity (phosphorylation) of p38α MAPK within the dorsal raphe nucleus (DRN), which sends serotonin (5HT)-containing projections to forebrain areas critical for modulating fear and anxiety responses (Lowry et al., 2005). This effect is mimicked by administration of U50,488, which produces dysphoria (see Carlezon et al., 2009), and is blocked by KOR antagonism. Activation of p38α MAPK leads to increases in surface expression of 5HT transporters and, in turn, decreases in extracellular levels of 5HT. Indeed, central administration of CRF inhibits DRN neurons and produces decreases in 5HT release (Price et al., 1998; Kirby et al., 2000). Thus it is possible that the effects of JDTic reported here may be due to inhibition of CRF-induced decreases in 5HT via blockade

of CRF-regulating KORs within the DRN (see Bruchas et al., 2011; Muschamp et al., 2011a). Another possibility is that actions in striatal regions may contribute to these effects. Lesions of the striatum, an area rich in dopamine (DA), degrade 5CSRTT performance (Rogers et al., 2001). Previous work has shown that systemic DA receptor antagonism decreases premature responses and increases omissions and response latencies in the 5CSRTT (Harrison et al., 1997). Similarly, depletion of DA from the striatum increases both response latencies and omissions (Cole and Robbins, 1989; Baunez and Robbins, 1999), a pattern of effects similar to that which we observed after CRF administration. CRF stimulates dynorphin release (Nikolarakis et al., 1986; Song and Takemori, 1992), which can in turn inhibit DA release via KOR activation at terminals of midbrain DA neurons (Donzanti et al., 1992; Svingos et al., 1999), creating a hypo-dopaminergic state. Additionally, there is evidence that the DRN sends direct projections to the striatum that regulate DA release: activation of 5HT receptors in the striatum increases extracellular concentrations of DA (Benloucif and Galloway, 1991; Benloucif et al., 1993) whereas CRF reduces 5HT release in the striatum (Price et al., 1998), raising the possibility that CRF-induced decreases in 5HT may reduce DA activity in this region. Blockade of KORs with JDTic either at DA nerve terminals or within the DRN may attenuate reductions in DA activity, thereby improving performance in the 5CSRTT. These possibilities are not mutually exclusive; indeed, each may be only one of several mechanisms acting in concert, considering that stress can elevate dynorphin expression in areas including the ventral striatum (nucleus accumbens [NAc]) and hippocampus, and that microinjections of KOR antagonists into these regions is sufficient to produce antidepressant-like effects (Pliakas et al., 2001; Newton et al., 2002; Shirayama et al., 2004; Muschamp et al., 2011b). The NAc is of particular

interest because it is embedded within a complex circuitry that can influence the function of other brain areas implicated in motivation and emotion, such as the frontal cortex and amygdala (see Carlezon and Thomas, 2009). It is not yet known if all of these effects can be tied together within a single neural circuitry model. Clearly, a broad scope of additional work is needed to further characterize CRF-KOR interactions and determine if effects in the various behavioral tests are attributable to a uniform neural substrate or circuit.

JDTic (10 mg/kg) was administered once, 24 hr before testing began, because this drug is known to have a slow onset and long duration of action (Carroll et al., 2004; Knoll et al. 2007; Knoll and Carlezon 2010). Indeed, data from the present study confirm that JDTic can disrupt responsiveness to a KOR-selective agonist for at least 11 days. The mechanism of this effect is not fully understood, but may involve ligand-directed signaling (also known as biased agonism), a process by which a drug can act as an antagonist of some downstream intracellular signaling pathways while simultaneously acting as an agonist at others. The long-lasting effects of KOR antagonists in general may be related to their ability to activate c-Jun N-terminal kinase-1 (JNK), leading to a de-coupling of KORs from their intracellular signaling cascades (Melief et al., 2010; Melief et al., 2011), rather than long-term persistence of these drugs in the brain (Munro et al., 2012). From a drug development perspective, such long-lasting effects may be ultimately desirable once safety and efficacy are established, but they complicate early-phase clinical studies in humans (Carlezon et al., 2009). Next-generation KOR antagonists that block p38α MAPK without stimulating JNK may be optimal as therapeutic agents.

These new findings supplement a growing body of evidence suggesting that KOR antagonists can block the effects of stress. These agents produce antidepressant-like effects in models that depend upon stressful experiences such as inescapable swimming or shock to trigger a depressive-like state (Pliakas et al., 2001; Newton et al., 2002; Mague et al., 2003). They also produce anxiolytic-like effects in models where stress produces a resistance to exploring open spaces (elevated plus maze) or persistent fear behaviors in the presence of cues associated with prior pain or trauma (fear conditioning) (Knoll et al., 2007). The fact that KOR antagonists produce both these antidepressant- and anxiolytic-like behaviors together gives them a unique profile, since acute administration of standard antidepressants tends to produce anxiogenic effects in rodent models that may reflect those often seen early in antidepressant treatment regimens in humans (Knoll et al., 2007).

There are currently no methods to prevent the immediate effects of stress or the subsequent development of anxiety or depressive disorders. In situations where stress can be predicted, the ability to intervene with a preventative measure in advance of stress exposure may promote short-term safety and long-term health. The present studies indicate that pretreatment with a KOR antagonist can improve cognitive performance that is typically degraded under stress-like conditions, an effect that would be particularly desirable in humans when stress is accompanied by danger or the potential for harm. Considering prior work showing that pretreatment with this same class of agents has anxiolytic effects and attenuates the development of conditioned fear (Knoll et al, 2007) in a rodent version of a method often used to study PTSD in people (Mahan and Ressler, 2012), these new findings provide further evidence that

KOR antagonists can prevent stress-induced processes that may render individuals vulnerable to acute injury and contribute to the development of psychiatric illness.

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Dr. Carlezon has a US patent covering the use of kappa antagonists in the treatment of depression (Assignee: McLean Hospital). In the last three years Dr. Carlezon has received compensation for professional services from The American College of Neuropsychopharmacology and Myneurolab.com. Dr. Carroll discloses that he and the Research Triangle Institute are co-owners of US patents claiming the composition of JDTic. Dr. Cohen has pending patents on pyrimidines to treat bipolar disorders, kappa-opioid agonists in bipolar mania, and mitochondrial replacement. Ms. Van't Veer and Ms. Yano have no disclosures.

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References

Abduljawad KA, Langley RW, Bradshaw CM, Szabadi E (2001) Effects of clonidine and diazepam on prepulse inhibition of the acoustic startle response and the N1/P2 auditory evoked potential in man. J Psychopharmacol 15:237-242.

Abercrombie ED, Keefe KA, DiFrischia DS, Zigmond MJ (1989) Differential effect of stress on in vivo dopamine release in striatum, nucleus accumbens, and medial frontal cortex. Journal of neurochemistry 52:1655-1658.

Ahima RS, Harlan RE (1990) Charting of type II glucocorticoid receptor-like immunoreactivity in the rat central nervous system. Neuroscience 39:579-604.

Ahmed BY, Chakravarthy S, Eggers R, Hermens WT, Zhang JY, Niclou SP, Levelt C, Sablitzky F, Anderson PN, Lieberman AR, Verhaagen J (2004) Efficient delivery of Cre-recombinase to neurons in vivo and stable transduction of neurons using adeno-associated and lentiviral vectors. BMC neuroscience 5:4.

Ahmed SH, Koob GF (2005) Transition to drug addiction: a negative reinforcement model based on an allostatic decrease in reward function. Psychopharmacology 180:473-490.

Aisa B, Tordera R, Lasheras B, Del Rio J, Ramirez MJ (2008) Effects of maternal separation on hypothalamic-pituitary-adrenal responses, cognition and vulnerability to stress in adult female rats. Neuroscience 154:1218-1226.

Aldrich JV, Patkar KA, McLaughlin JP (2009) Zyklophin, a systemically active selective kappa opioid receptor peptide antagonist with short duration of action. Proceedings of the National Academy of Sciences of the United States of America 106:18396-18401.

Almeida OF, Nikolarakis KE, Herz A (1986) Regulation of hypothalamic beta-endorphin and dynorphin release by corticotropin-releasing factor (CRF). NIDA research monograph 75:401-402.

Alon T, Zhou L, Perez CA, Garfield AS, Friedman JM, Heisler LK (2009) Transgenic mice expressing green fluorescent protein under the control of the corticotropin-releasing hormone promoter. Endocrinology 150:5626-5632.

Amaral DG (1978) A Golgi study of cell types in the hilar region of the hippocampus in the rat. The Journal of comparative neurology 182:851-914.

American Psychiatric Association (2000) Diagnostic and statistical manual of mental disorders, 4th Edition. Washington, DC: American Psychiatric Association.

Anagnostaras SG, Maren S, Fanselow MS (1999) Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: within-subjects examination. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:1106-1114.

Andreae LC, Herbert J (1993) Expression of c-fos in restricted areas of the basal forebrain and brainstem following single or combined intraventricular infusions of vasopressin and corticotropin-releasing factor. Neuroscience 53:735-748.

Antoni FA (1986) Hypothalamic control of adrenocorticotropin secretion: advances since the discovery of 41-residue corticotropin-releasing factor. Endocrine reviews 7:351-378.

Arato M, Banki CM, Bissette G, Nemeroff CB (1989) Elevated CSF CRF in suicide victims. Biological psychiatry 25:355-359.

Arborelius L, Owens MJ, Plotsky PM, Nemeroff CB (1999) The role of corticotropin-releasing factor in depression and anxiety disorders. J Endocrinol 160:1-12.

Attali B, Saya D, Vogel Z (1989) Kappa-opiate agonists inhibit adenylate cyclase and produce heterologous desensitization in rat spinal cord. Journal of neurochemistry 52:360-369.

Aubry JM, Pozzoli G, Vale WW (1999) Chronic treatment with the antidepressant amitriptyline decreases CRF-R1 receptor mRNA levels in the rat amygdala. Neuroscience letters 266:197-200.

Autelitano DJ, Blum M, Lopingco M, Allen RG, Roberts JL (1990) Corticotropin-releasing factor differentially regulates anterior and intermediate pituitary lobe proopiomelanocortin gene transcription, nuclear precursor RNA and mature mRNA in vivo. Neuroendocrinology 51:123-130.

Avgustinovich DF, Kovalenko IL, Kudryavtseva NN (2005) A model of anxious depression: persistence of behavioral pathology. Neuroscience and behavioral physiology 35:917-924.

Baker DG, West SA, Nicholson WE, Ekhator NN, Kasckow JW, Hill KK, Bruce AB, Orth DN, Geracioti TD, Jr. (1999) Serial CSF corticotropin-releasing hormone levels and adrenocortical activity in combat veterans with posttraumatic stress disorder. The American journal of psychiatry 156:585-588.

Bakshi VP, Smith-Roe S, Newman SM, Grigoriadis DE, Kalin NH (2002) Reduction of stress-induced behavior by antagonism of corticotropin-releasing hormone 2 (CRH2) receptors in lateral septum or CRH1 receptors in amygdala. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:2926-2935.

Bale TL, Lee KF, Vale WW (2002) The role of corticotropin-releasing factor receptors in stress and anxiety. Integrative and comparative biology 42:552-555.

Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE, Koob GF, Vale WW, Lee KF (2000) Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. Nature genetics 24:410-414.

Ballenger JC (1999) Current treatments of the anxiety disorders in adults. Biological psychiatry 46:1579-1594.

Bals-Kubik R, Ableitner A, Herz A, Shippenberg TS (1993) Neuroanatomical sites mediating the motivational effects of opioids as mapped by the conditioned place preference paradigm in rats. The Journal of pharmacology and experimental therapeutics 264:489-495.

Bangasser DA, Valentino RJ (2012) Sex differences in molecular and cellular substrates of stress. Cellular and molecular neurobiology 32:709-723.

Bannerman DM, Deacon RM, Offen S, Friswell J, Grubb M, Rawlins JN (2002) Double dissociation of function within the hippocampus: spatial memory and hyponeophagia. Behavioral neuroscience 116:884-901.

Barrientos RM, O'Reilly RC, Rudy JW (2002) Memory for context is impaired by injecting anisomycin into dorsal hippocampus following context exploration. Behavioural brain research 134:299-306.

Barrot M, Wallace DL, Bolanos CA, Graham DL, Perrotti LI, Neve RL, Chambliss H, Yin JC, Nestler EJ (2005) Regulation of anxiety and initiation of sexual behavior by CREB in

the nucleus accumbens. Proceedings of the National Academy of Sciences of the United States of America 102:8357-8362.

Barrot M, Olivier JD, Perrotti LI, DiLeone RJ, Berton O, Eisch AJ, Impey S, Storm DR, Neve RL, Yin JC, Zachariou V, Nestler EJ (2002) CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. Proceedings of the National Academy of Sciences of the United States of America 99:11435-11440.

Bassareo V, De Luca MA, Di Chiara G (2002) Differential Expression of Motivational Stimulus Properties by Dopamine in Nucleus Accumbens Shell versus Core and Prefrontal Cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:4709-4719.

Baunez C, Robbins TW (1999) Effects of dopamine depletion of the dorsal striatum and further interaction with subthalamic nucleus lesions in an attentional task in the rat. Neuroscience 92:1343-1356.

Beardsley PM, Howard JL, Shelton KL, Carroll FI (2005) Differential effects of the novel kappa opioid receptor antagonist, JDTic, on reinstatement of cocaine-seeking induced by footshock stressors vs cocaine primes and its antidepressant-like effects in rats. Psychopharmacology 183:118-126.

Beaulieu S, Di Paolo T, Cote J, Barden N (1987) Participation of the central amygdaloid nucleus in the response of adrenocorticotropin secretion to immobilization stress: opposing roles of the noradrenergic and dopaminergic systems. Neuroendocrinology 45:37-46.

Beck CH, Fibiger HC (1995) Conditioned fear-induced changes in behavior and in the expression of the immediate early gene c-fos: with and without diazepam pretreatment. The Journal of neuroscience : the official journal of the Society for Neuroscience 15:709-720.

Beck LH, Bransome ED, Jr., Mirsky AF, Rosvold HE, Sarason I (1956) A continuous performance test of brain damage. Journal of consulting psychology 20:343-350.

Béguin C, Cohen BM (2009) Medicinal Chemistry of Kappa Opioid Receptor Antagonists. In: Opiate Receptors and Antagonists From Bench to Clinic (Dean RL, Bilsky EJ, Negus SS, eds), pp 99-118 Totowa, NJ: Humana Press,.

Belcheva MM, Clark AL, Haas PD, Serna JS, Hahn JW, Kiss A, Coscia CJ (2005) Mu and kappa opioid receptors activate ERK/MAPK via different protein kinase C isoforms and secondary messengers in astrocytes. The Journal of biological chemistry 280:27662-27669.

Belcheva MM, Vogel Z, Ignatova E, Avidor-Reiss T, Zippel R, Levy R, Young EC, Barg J, Coscia CJ (1998) Opioid modulation of extracellular signal-regulated protein kinase activity is ras-dependent and involves Gbetagamma subunits. Journal of neurochemistry 70:635-645.

Benazzouz A, Hallett M (2000) Mechanism of action of deep brain stimulation. Neurology 55:S13-16.

Benloucif S, Galloway MP (1991) Facilitation of dopamine release in vivo by serotonin agonists: studies with microdialysis. European journal of pharmacology 200:1-8.

Benloucif S, Keegan MJ, Galloway MP (1993) Serotonin-facilitated dopamine release in vivo: pharmacological characterization. The Journal of pharmacology and experimental therapeutics 265:373-377.

Berretta S, Pantazopoulos H, Caldera M, Pantazopoulos P, Pare D (2005) Infralimbic cortex activation increases c-Fos expression in intercalated neurons of the amygdala. Neuroscience 132:943-953.

Berton O, McClung CA, Dileone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM, Bolanos CA, Rios M, Monteggia LM, Self DW, Nestler EJ (2006) Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. Science 311:864-868.

Bevins RA (2005) The Reference-Dose Place Conditioning Procedure Yields a Graded Dose-Effect Function. International Journal of Comparative Psychology 18:101-111.

Bewernick BH, Kayser S, Sturm V, Schlaepfer TE (2012) Long-term effects of nucleus accumbens deep brain stimulation in treatment-resistant depression: evidence for sustained efficacy. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 37:1975-1985.

Bewernick BH, Hurlemann R, Matusch A, Kayser S, Grubert C, Hadrysiewicz B, Axmacher N, Lemke M, Cooper-Mahkorn D, Cohen MX, Brockmann H, Lenartz D, Sturm V, Schlaepfer TE (2010) Nucleus accumbens deep brain stimulation decreases

ratings of depression and anxiety in treatment-resistant depression. Biological psychiatry 67:110-116.

Bhatnagar S, Dallman M (1998) Neuroanatomical basis for facilitation of hypothalamic-pituitary-adrenal responses to a novel stressor after chronic stress. Neuroscience 84:1025-1039.

Bittencourt JC, Sawchenko PE (2000) Do centrally administered neuropeptides access cognate receptors?: an analysis in the central corticotropin-releasing factor system. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:1142-1156.

Blank T, Nijholt I, Grammatopoulos DK, Randeva HS, Hillhouse EW, Spiess J (2003) Corticotropin-releasing factor receptors couple to multiple G-proteins to activate diverse intracellular signaling pathways in mouse hippocampus: role in neuronal excitability and associative learning. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:700-707.

Blond S, Caparros-Lefebvre D, Parker F, Assaker R, Petit H, Guieu JD, Christiaens JL (1992) Control of tremor and involuntary movement disorders by chronic stereotactic stimulation of the ventral intermediate thalamic nucleus. Journal of neurosurgery 77:62-68.

Bohn LM, Belcheva MM, Coscia CJ (2000) Mitogenic signaling via endogenous kappa-opioid receptors in C6 glioma cells: evidence for the involvement of protein kinase C and the mitogen-activated protein kinase signaling cascade. Journal of neurochemistry 74:564-573.

Borowski TB, Kokkinidis L (1996) Contribution of ventral tegmental area dopamine neurons to expression of conditional fear: effects of electrical stimulation, excitotoxin lesions, and quinpirole infusion on potentiated startle in rats. Behavioral neuroscience 110:1349-1364.

Borowski TB, Kokkinidis L (1998) The effects of cocaine, amphetamine, and the dopamine D1 receptor agonist SKF 38393 on fear extinction as measured with potentiated startle: implications for psychomotor stimulant psychosis. Behavioral neuroscience 112:952-965.

Bortolato M, Aru GN, Frau R, Orru M, Fa M, Manunta M, Puddu M, Mereu G, Gessa GL (2005) Kappa opioid receptor activation disrupts prepulse inhibition of the acoustic startle in rats. Biological psychiatry 57:1550-1558.

Bossini L, Tavanti M, Calossi S, Lombardelli A, Polizzotto NR, Galli R, Vatti G, Pieraccini F, Castrogiovanni P (2008) Magnetic resonance imaging volumes of the hippocampus in drug-naive patients with post-traumatic stress disorder without comorbidity conditions. Journal of psychiatric research 42:752-762.

Boulis NM, Davis M (1989) Footshock-induced sensitization of electrically elicited startle reflexes. Behavioral neuroscience 103:504-508.

Brady LS, Whitfield HJ, Jr., Fox RJ, Gold PW, Herkenham M (1991) Long-term antidepressant administration alters corticotropin-releasing hormone, tyrosine hydroxylase, and mineralocorticoid receptor gene expression in rat brain. Therapeutic implications. The Journal of clinical investigation 87:831-837.

Brady LS, Gold PW, Herkenham M, Lynn AB, Whitfield HJ, Jr. (1992) The antidepressants fluoxetine, idazoxan and phenelzine alter corticotropin-releasing hormone and tyrosine hydroxylase mRNA levels in rat brain: therapeutic implications. Brain research 572:117-125.

Bremner JD, Licinio J, Darnell A, Krystal JH, Owens MJ, Southwick SM, Nemeroff CB, Charney DS (1997a) Elevated CSF corticotropin-releasing factor concentrations in posttraumatic stress disorder. The American journal of psychiatry 154:624-629.

Bremner JD, Randall P, Scott TM, Bronen RA, Seibyl JP, Southwick SM, Delaney RC, McCarthy G, Charney DS, Innis RB (1995) MRI-based measurement of hippocampal volume in patients with combat-related posttraumatic stress disorder. The American journal of psychiatry 152:973-981.

Bremner JD, Randall P, Vermetten E, Staib L, Bronen RA, Mazure C, Capelli S, McCarthy G, Innis RB, Charney DS (1997b) Magnetic resonance imaging-based measurement of hippocampal volume in posttraumatic stress disorder related to childhood physical and sexual abuse--a preliminary report. Biological psychiatry 41:23-32.

Bremner JD, Vythilingam M, Vermetten E, Southwick SM, McGlashan T, Nazeer A, Khan S, Vaccarino LV, Soufer R, Garg PK, Ng CK, Staib LH, Duncan JS, Charney DS (2003) MRI and PET study of deficits in hippocampal structure and function in women with childhood sexual abuse and posttraumatic stress disorder. The American journal of psychiatry 160:924-932.

Britton DR, Koob GF, Rivier J, Vale W (1982) Intraventricular corticotropin-releasing factor enhances behavioral effects of novelty. Life sciences 31:363-367.

Britton KT, Lee G, Vale W, Rivier J, Koob GF (1986) Corticotropin releasing factor (CRF) receptor antagonist blocks activating and 'anxiogenic' actions of CRF in the rat. Brain research 369:303-306.

Bruchas MR, Chavkin C (2010) Kinase cascades and ligand-directed signaling at the kappa opioid receptor. Psychopharmacology 210:137-147.

Bruchas MR, Land BB, Chavkin C (2010) The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. Brain research 1314:44-55.

Bruchas MR, Macey TA, Lowe JD, Chavkin C (2006) Kappa opioid receptor activation of p38 MAPK is GRK3- and arrestin-dependent in neurons and astrocytes. The Journal of biological chemistry 281:18081-18089.

Bruchas MR, Land BB, Lemos JC, Chavkin C (2009) CRF1-R activation of the dynorphin/kappa opioid system in the mouse basolateral amygdala mediates anxiety-like behavior. PloS one 4:e8528.

Bruchas MR, Land BB, Aita M, Xu M, Barot SK, Li S, Chavkin C (2007a) Stress-induced p38 mitogen-activated protein kinase activation mediates kappa-opioid-dependent dysphoria. The Journal of neuroscience : the official journal of the Society for Neuroscience 27:11614-11623.

Bruchas MR, Yang T, Schreiber S, Defino M, Kwan SC, Li S, Chavkin C (2007b) Long-acting kappa opioid antagonists disrupt receptor signaling and produce noncompetitive effects by activating c-Jun N-terminal kinase. The Journal of biological chemistry 282:29803-29811.

Bruchas MR, Schindler AG, Shankar H, Messinger DI, Miyatake M, Land BB, Lemos JC, Hagan CE, Neumaier JF, Quintana A, Palmiter RD, Chavkin C (2011) Selective p38alpha MAPK deletion in serotonergic neurons produces stress resilience in models of depression and addiction. Neuron 71:498-511.

Bruijnzeel AW (2009) kappa-Opioid receptor signaling and brain reward function. Brain research reviews 62:127-146.

Buckmaster PS, Schwartzkroin PA (1994) Hippocampal mossy cell function: a speculative view. Hippocampus 4:393-402.

Caine SB, Geyer MA, Swerdlow NR (1992) Hippocampal modulation of acoustic startle and prepulse inhibition in the rat. Pharmacology, biochemistry, and behavior 43:1201-1208.

Campeau S, Liberzon I, Morilak D, Ressler K (2011) Stress modulation of cognitive and affective processes. Stress 14:503-519.

Canteras NS, Simerly RB, Swanson LW (1995) Organization of projections from the medial nucleus of the amygdala: a PHAL study in the rat. The Journal of comparative neurology 360:213-245.

Cao JL, Covington HE, 3rd, Friedman AK, Wilkinson MB, Walsh JJ, Cooper DC, Nestler EJ, Han MH (2010) Mesolimbic dopamine neurons in the brain reward circuit mediate susceptibility to social defeat and antidepressant action. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:16453-16458.

Carey AN, Borozny K, Aldrich JV, McLaughlin JP (2007) Reinstatement of cocaine place-conditioning prevented by the peptide kappa-opioid receptor antagonist arodyn. European journal of pharmacology 569:84-89.

Carey AN, Lyons AM, Shay CF, Dunton O, McLaughlin JP (2009) Endogenous kappa opioid activation mediates stress-induced deficits in learning and memory. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:4293-4300.

Carlezon WA, Jr. (2003) Place conditioning to study drug reward and aversion. Methods in molecular medicine 84:243-249.

Carlezon WA, Jr., Nestler EJ (2002) Elevated levels of GluR1 in the midbrain: a trigger for sensitization to drugs of abuse? Trends in neurosciences 25:610-615.

Carlezon WA, Jr., Chartoff EH (2007) Intracranial self-stimulation (ICSS) in rodents to study the neurobiology of motivation. Nat Protoc 2:2987-2995.

Carlezon WA, Jr., Thomas MJ (2009) Biological substrates of reward and aversion: a nucleus accumbens activity hypothesis. Neuropharmacology 56 Suppl 1:122-132.

Carlezon WA, Jr., Carroll FI (in-press) Development of Kappa Opioid Receptor Antagonists. Journal of medicinal chemistry.

Carlezon WA, Jr., Beguin C, Knoll AT, Cohen BM (2009) Kappa-opioid ligands in the study and treatment of mood disorders. Pharmacology & therapeutics 123:334-343.

Carlezon WA, Jr., Thome J, Olson VG, Lane-Ladd SB, Brodkin ES, Hiroi N, Duman RS, Neve RL, Nestler EJ (1998) Regulation of cocaine reward by CREB. Science 282:2272-2275.

Carlezon WA, Jr., Beguin C, DiNieri JA, Baumann MH, Richards MR, Todtenkopf MS, Rothman RB, Ma Z, Lee DY, Cohen BM (2006) Depressive-like effects of the kappa-opioid receptor agonist salvinorin A on behavior and neurochemistry in rats. The Journal of pharmacology and experimental therapeutics 316:440-447.

Carlsen J, Heimer L (1988) The basolateral amygdaloid complex as a cortical-like structure. Brain research 441:377-380.

Carr GV, Lucki I (2010) Comparison of the kappa-opioid receptor antagonist DIPPA in tests of anxiety-like behavior between Wistar Kyoto and Sprague Dawley rats. Psychopharmacology 210:295-302.

Carr GV, Bangasser DA, Bethea T, Young M, Valentino RJ, Lucki I (2010) Antidepressant-like effects of kappa-opioid receptor antagonists in Wistar Kyoto rats. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 35:752-763.

Carroll I, Thomas JB, Dykstra LA, Granger AL, Allen RM, Howard JL, Pollard GT, Aceto MD, Harris LS (2004) Pharmacological properties of JDTic: a novel kappa-opioid receptor antagonist. European journal of pharmacology 501:111-119.

Chabot CC, Taylor DH (1992) Daily rhythmicity of the rat acoustic startle response. Physiology & behavior 51:885-889.

Chapman WP, Schroeder HR, Geyer G, Brazier MA, Fager C, Poppen JL, Solomon HC, Yakovlev PI (1954) Physiological evidence concerning importance of the amygdaloid nuclear region in the integration of circulatory function and emotion in man. Science 120:949-950.

Chartoff EH, Papadopoulou M, MacDonald ML, Parsegian A, Potter D, Konradi C, Carlezon WA, Jr. (2009) Desipramine reduces stress-activated dynorphin expression and CREB phosphorylation in NAc tissue. Molecular pharmacology 75:704-712.

Chavkin C, James IF, Goldstein A (1982) Dynorphin is a specific endogenous ligand of the kappa opioid receptor. Science 215:413-415.

Chefer VI, Czyzyk T, Bolan EA, Moron J, Pintar JE, Shippenberg TS (2005) Endogenous kappa-opioid receptor systems regulate mesoaccumbal dopamine dynamics and vulnerability to cocaine. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:5029-5037.

Chen YW, Rada PV, Butzler BP, Leibowitz SF, Hoebel BG (2012) Corticotropin-releasing factor in the nucleus accumbens shell induces swim depression, anxiety, and anhedonia along with changes in local dopamine/acetylcholine balance. Neuroscience 206:155-166.

Chiba T, Kayahara T, Nakano K (2001) Efferent projections of infralimbic and prelimbic areas of the medial prefrontal cortex in the Japanese monkey, Macaca fuscata. Brain research 888:83-101.

Chilcoat HD, Breslau N (1998) Investigations of causal pathways between PTSD and drug use disorders. Addictive behaviors 23:827-840.

Cintra A, Zoli M, Rosen L, Agnati LF, Okret S, Wikstrom AC, Gustaffsson JA, Fuxe K (1994) Mapping and computer assisted morphometry and microdensitometry of glucocorticoid receptor immunoreactive neurons and glial cells in the rat central nervous system. Neuroscience 62:843-897.

Code RA, Fallon JH (1986) Some projections of dynorphin-immunoreactive neurons in the rat central nervous system. Neuropeptides 8:165-172.

Cohen BM, Murphy B (2008) The effects of pentazocine, a kappa agonist, in patients with mania. Int J Neuropsychopharmacol 11:243-247.

Cole BJ, Robbins TW (1989) Effects of 6-hydroxydopamine lesions of the nucleus accumbens septi on performance of a 5-choice serial reaction time task in rats: implications for theories of selective attention and arousal. Behavioural brain research 33:165-179.

Cole RL, Konradi C, Douglass J, Hyman SE (1995) Neuronal adaptation to amphetamine and dopamine: molecular mechanisms of prodynorphin gene regulation in rat striatum. Neuron 14:813-823.

Coover GD, Levine S (1972) Auditory startle response of hippocampectomized rats. Physiology & behavior 9:75-77.

Corcoran KA, Desmond TJ, Frey KA, Maren S (2005) Hippocampal inactivation disrupts the acquisition and contextual encoding of fear extinction. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:8978-8987.

Covington HE, 3rd, Lobo MK, Maze I, Vialou V, Hyman JM, Zaman S, LaPlant Q, Mouzon E, Ghose S, Tamminga CA, Neve RL, Deisseroth K, Nestler EJ (2010) Antidepressant effect of optogenetic stimulation of the medial prefrontal cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience 30:16082-16090.

Crane JW, Ebner K, Day TA (2003) Medial prefrontal cortex suppression of the hypothalamic-pituitary-adrenal axis response to a physical stressor, systemic delivery of interleukin-1beta. The European journal of neuroscience 17:1473-1481.

Crawley JN (2007) What's wrong with my mouse? : behavioral phenotyping of transgenic and knockout mice, 2nd Edition. Hoboken: John Wiley.

Creighton TE (1993) Proteins : structures and molecular properties, 2nd Edition. New York: W.H. Freeman.

Cryan JF, Markou A, Lucki I (2002) Assessing antidepressant activity in rodents: recent developments and future needs. Trends in pharmacological sciences 23:238-245.

Cullinan WE, Herman JP, Watson SJ (1993) Ventral subicular interaction with the hypothalamic paraventricular nucleus: evidence for a relay in the bed nucleus of the stria terminalis. The Journal of comparative neurology 332:1-20.

Dalman FC, O'Malley KL (1999) kappa-Opioid tolerance and dependence in cultures of dopaminergic midbrain neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:5750-5757.

Dautzenberg FM, Hauger RL (2002) The CRF peptide family and their receptors: yet more partners discovered. Trends in pharmacological sciences 23:71-77.

Davis HP, Squire LR (1984) Protein synthesis and memory: a review. Psychological bulletin 96:518-559.

Davis M (1979) Diazepam and flurazepam: effects on conditioned fear as measured with the potentiated startle paradigm. Psychopharmacology 62:1-7.

Davis M (1989) Sensitization of the acoustic startle reflex by footshock. Behavioral neuroscience 103:495-503.

Davis M (1993) Pharmacological analysis of fear-potentiated startle. Braz J Med Biol Res 26:235-260.

Davis M (1997) Neurobiology of fear responses: the role of the amygdala. The Journal of neuropsychiatry and clinical neurosciences 9:382-402.

Davis M (2002) Neural circuitry of anxiety and stress disorders. In: Neuropsychopharmacology : the fifth generation of progress : an official publication of the American College of Neuropsychopharmacology (Davis KL, American College of Neuropsychopharmacology., eds), pp xxi, 2010 p. Philadelphia: Lippincott/Williams & Wilkins.

Davis M, Astrachan DI (1978) Conditioned fear and startle magnitude: effects of different footshock or backshock intensities used in training. Journal of experimental psychology Animal behavior processes 4:95-103.

Davis M, Shi C (2000) The amygdala. Current Biology 10:R131.

Davis M, Walker DL, Lee Y (1997) Roles of the amygdala and bed nucleus of the stria terminalis in fear and anxiety measured with the acoustic startle reflex. Possible relevance to PTSD. Annals of the New York Academy of Sciences 821:305-331.

Davis M, Gendelman DS, Tischler MD, Gendelman PM (1982) A primary acoustic startle circuit: lesion and stimulation studies. The Journal of neuroscience : the official journal of the Society for Neuroscience 2:791-805.

Davis M, Walker DL, Miles L, Grillon C (2010) Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 35:105-135.

Dazzi L, Serra M, Spiga F, Pisu MG, Jentsch JD, Biggio G (2001) Prevention of the stress-induced increase in frontal cortical dopamine efflux of freely moving rats by long-

term treatment with antidepressant drugs. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology 11:343-349.

de Kloet CS, Vermetten E, Geuze E, Lentjes EG, Heijnen CJ, Stalla GK, Westenberg HG (2008) Elevated plasma corticotrophin-releasing hormone levels in veterans with posttraumatic stress disorder. Progress in brain research 167:287-291.

De Souza EB (1995) Corticotropin-releasing factor receptors: physiology, pharmacology, biochemistry and role in central nervous system and immune disorders. Psychoneuroendocrinology 20:789-819.

De Souza EB, Insel TR, Perrin MH, Rivier J, Vale WW, Kuhar MJ (1985) Corticotropin-releasing factor receptors are widely distributed within the rat central nervous system: an autoradiographic study. The Journal of neuroscience : the official journal of the Society for Neuroscience 5:3189-3203.

Deak T, Nguyen KT, Ehrlich AL, Watkins LR, Spencer RL, Maier SF, Licinio J, Wong ML, Chrousos GP, Webster E, Gold PW (1999) The impact of the nonpeptide corticotropin-releasing hormone antagonist antalarmin on behavioral and endocrine responses to stress. Endocrinology 140:79-86.

Deshmukh SS, Bhalla US (2003) Representation of odor habituation and timing in the hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:1903-1915.

Detke MJ, Rickels M, Lucki I (1995) Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. Psychopharmacology 121:66-72.

Deutch AY, Roth RH (1990) The determinants of stress-induced activation of the prefrontal cortical dopamine system. Progress in brain research 85:367-402; discussion 402-363.

Deutch AY, Tam SY, Roth RH (1985) Footshock and conditioned stress increase 3,4-dihydroxyphenylacetic acid (DOPAC) in the ventral tegmental area but not substantia nigra. Brain research 333:143-146.

Devine DP, Leone P, Pocock D, Wise RA (1993) Differential involvement of ventral tegmental mu, delta and kappa opioid receptors in modulation of basal mesolimbic

dopamine release: in vivo microdialysis studies. The Journal of pharmacology and experimental therapeutics 266:1236-1246.

Di Chiara G, Imperato A (1988) Opposite effects of mu and kappa opiate agonists on dopamine release in the nucleus accumbens and in the dorsal caudate of freely moving rats. The Journal of pharmacology and experimental therapeutics 244:1067-1080.

Dinieri JA, Nemeth CL, Parsegian A, Carle T, Gurevich VV, Gurevich E, Neve RL, Nestler EJ, Carlezon WA, Jr. (2009) Altered sensitivity to rewarding and aversive drugs in mice with inducible disruption of cAMP response element-binding protein function within the nucleus accumbens. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:1855-1859.

Dong HW, Swanson LW (2004) Projections from bed nuclei of the stria terminalis, posterior division: implications for cerebral hemisphere regulation of defensive and reproductive behaviors. The Journal of comparative neurology 471:396-433.

Dong HW, Petrovich GD, Swanson LW (2001a) Topography of projections from amygdala to bed nuclei of the stria terminalis. Brain research Brain research reviews 38:192-246.

Dong HW, Petrovich GD, Watts AG, Swanson LW (2001b) Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain. The Journal of comparative neurology 436:430-455.

Donzanti BA, Althaus JS, Payson MM, Von Voigtlander PF (1992) Kappa agonist-induced reduction in dopamine release: site of action and tolerance. Research communications in chemical pathology and pharmacology 78:193-210.

Douglass J, McKinzie AA, Pollock KM (1994) Identification of multiple DNA elements regulating basal and protein kinase A-induced transcriptional expression of the rat prodynorphin gene. Mol Endocrinol 8:333-344.

Duncan GE, Knapp DJ, Breese GR (1996) Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. Brain research 713:79-91.

Dunn AJ, File SE (1987) Corticotropin-releasing factor has an anxiogenic action in the social interaction test. Horm Behav 21:193-202.

Dunn AJ, Berridge CW (1990) Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? Brain research Brain research reviews 15:71-100.

Durant C, Christmas D, Nutt D (2010) The pharmacology of anxiety. Current topics in behavioral neurosciences 2:303-330.

Ebner SR, Roitman MF, Potter DN, Rachlin AB, Chartoff EH (2010) Depressive-like effects of the kappa opioid receptor agonist salvinorin A are associated with decreased phasic dopamine release in the nucleus accumbens. Psychopharmacology 210:241-252.

Eckart K, Jahn O, Radulovic J, Radulovic M, Blank T, Stiedl O, Brauns O, Tezval H, Zeyda T, Spiess J (2002) Pharmacology and biology of corticotropin-releasing factor (CRF) receptors. Receptors Channels 8:163-177.

Endoh T, Matsuura H, Tanaka C, Nagase H (1992) Nor-binaltorphimine: a potent and selective kappa-opioid receptor antagonist with long-lasting activity in vivo. Archives internationales de pharmacodynamie et de therapie 316:30-42.

Espejo EF (1997) Selective dopamine depletion within the medial prefrontal cortex induces anxiogenic-like effects in rats placed on the elevated plus maze. Brain research 762:281-284.

Fadda P, Pani L, Porcella A, Fratta W (1995) Chronic imipramine, L-sulpiride and mianserin decrease corticotropin releasing factor levels in the rat brain. Neuroscience letters 192:121-123.

Fallon JH, Leslie FM (1986) Distribution of dynorphin and enkephalin peptides in the rat brain. The Journal of comparative neurology 249:293-336.

Feenstra MG, Vogel M, Botterblom MH, Joosten RN, de Bruin JP (2001) Dopamine and noradrenaline efflux in the rat prefrontal cortex after classical aversive conditioning to an auditory cue. The European journal of neuroscience 13:1051-1054.

Feil S, Valtcheva N, Feil R (2009) Inducible Cre mice. Methods in molecular biology 530:343-363.

Fernandez Espejo E (2003) Prefrontocortical dopamine loss in rats delays long-term extinction of contextual conditioned fear, and reduces social interaction without affecting short-term social interaction memory. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 28:490-498.

Filliol D, Ghozland S, Chluba J, Martin M, Matthes HW, Simonin F, Befort K, Gaveriaux-Ruff C, Dierich A, LeMeur M, Valverde O, Maldonado R, Kieffer BL (2000) Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. Nature genetics 25:195-200.

Fisahn A, Lavebratt C, Canlon B (2011) Acoustic startle hypersensitivity in Mceph mice and its effect on hippocampal excitability. The European journal of neuroscience 34:1121-1130.

Flint J, Corley R, DeFries JC, Fulker DW, Gray JA, Miller S, Collins AC (1995) A simple genetic basis for a complex psychological trait in laboratory mice. Science 269:1432-1435.

Ford CP, Mark GP, Williams JT (2006) Properties and opioid inhibition of mesolimbic dopamine neurons vary according to target location. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:2788-2797.

Ford CP, Beckstead MJ, Williams JT (2007) Kappa opioid inhibition of somatodendritic dopamine inhibitory postsynaptic currents. Journal of neurophysiology 97:883-891.

Fox HC, Bergquist KL, Hong KI, Sinha R (2007) Stress-induced and alcohol cue-induced craving in recently abstinent alcohol-dependent individuals. Alcoholism, clinical and experimental research 31:395-403.

Fox MW (1965) The visual cliff test for the study of visual depth perception in the mouse. Animal behaviour 13:232-233.

Fraser HF, Rosenberg DE (1964) Studies on the Human Addiction Liability of 2'-Hydroxy-5-9-Dimethyl-2-(3,3-Dimethylallyl)-6,7-Benzomorphan (Win 20,228): A Weak Narcotic Antagonist. The Journal of pharmacology and experimental therapeutics 143:149-156.

Fujita M, Shimada S, Nishimura T, Uhl GR, Tohyama M (1993) Ontogeny of dopamine transporter mRNA expression in the rat brain. Brain research Molecular brain research 19:222-226.

Funk D, Li Z, Le AD (2006) Effects of environmental and pharmacological stressors on c-fos and corticotropin-releasing factor mRNA in rat brain: Relationship to the reinstatement of alcohol seeking. Neuroscience 138:235-243.

Funk D, Li Z, Shaham Y, Le AD (2003) Effect of blockade of corticotropin-releasing factor receptors in the median raphe nucleus on stress-induced c-fos mRNA in the rat brain. Neuroscience 122:1-4.

Fuster JM (2008) The prefrontal cortex, 4th Edition. Amsterdam ; Boston: Academic Press/Elsevier.

Fuxe K, Wikstrom AC, Okret S, Agnati LF, Harfstrand A, Yu ZY, Granholm L, Zoli M, Vale W, Gustafsson JA (1985) Mapping of glucocorticoid receptor immunoreactive neurons in the rat tel- and diencephalon using a monoclonal antibody against rat liver glucocorticoid receptor. Endocrinology 117:1803-1812.

Gambarana C, Masi F, Tagliamonte A, Scheggi S, Ghiglieri O, De Montis MG (1999) A chronic stress that impairs reactivity in rats also decreases dopaminergic transmission in the nucleus accumbens: a microdialysis study. Journal of neurochemistry 72:2039-2046.

Ghashghaei HT, Barbas H (2002) Pathways for emotion: interactions of prefrontal and anterior temporal pathways in the amygdala of the rhesus monkey. Neuroscience 115:1261-1279.

Ghiglieri O, Gambarana C, Scheggi S, Tagliamonte A, Willner P, De Montis MG (1997) Palatable food induces an appetitive behaviour in satiated rats which can be inhibited by chronic stress. Behavioural pharmacology 8:619-628.

Gifkins A, Greba Q, Kokkinidis L (2002) Ventral tegmental area dopamine neurons mediate the shock sensitization of acoustic startle: a potential site of action for benzodiazepine anxiolytics. Behavioral neuroscience 116:785-794.

Gilbertson MW, Shenton ME, Ciszewski A, Kasai K, Lasko NB, Orr SP, Pitman RK (2002) Smaller hippocampal volume predicts pathologic vulnerability to psychological trauma. Nature neuroscience 5:1242-1247.

Gillies GE, Linton EA, Lowry PJ (1982) Corticotropin releasing activity of the new CRF is potentiated several times by vasopressin. Nature 299:355-357.

Glick SD, Maisonneuve IM, Raucci J, Archer S (1995) Kappa opioid inhibition of morphine and cocaine self-administration in rats. Brain research 681:147-152.

Gonzalez LE, Ouagazzal AM, File SE (1998) Stimulation of benzodiazepine receptors in the dorsal hippocampus and median raphe reveals differential GABAergic control in two animal tests of anxiety. The European journal of neuroscience 10:3673-3680.

Gould E, McEwen BS, Tanapat P, Galea LA, Fuchs E (1997) Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:2492-2498.

Gould E, Tanapat P, McEwen BS, Flugge G, Fuchs E (1998) Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proceedings of the National Academy of Sciences of the United States of America 95:3168-3171.

Graham DL, Edwards S, Bachtell RK, DiLeone RJ, Rios M, Self DW (2007) Dynamic BDNF activity in nucleus accumbens with cocaine use increases self-administration and relapse. Nature neuroscience 10:1029-1037.

Graham DL, Krishnan V, Larson EB, Graham A, Edwards S, Bachtell RK, Simmons D, Gent LM, Berton O, Bolanos CA, DiLeone RJ, Parada LF, Nestler EJ, Self DW (2009) Tropomyosin-related kinase B in the mesolimbic dopamine system: region-specific effects on cocaine reward. Biological psychiatry 65:696-701.

Grammatopoulos DK, Randeva HS, Levine MA, Kanellopoulou KA, Hillhouse EW (2001) Rat cerebral cortex corticotropin-releasing hormone receptors: evidence for receptor coupling to multiple G-proteins. Journal of neurochemistry 76:509-519.

Gray TS, Carney ME, Magnuson DJ (1989) Direct projections from the central amygdaloid nucleus to the hypothalamic paraventricular nucleus: possible role in stress-induced adrenocorticotropin release. Neuroendocrinology 50:433-446.

Greba Q, Kokkinidis L (2000) Peripheral and intraamygdalar administration of the dopamine D1 receptor antagonist SCH 23390 blocks fear-potentiated startle but not shock reactivity or the shock sensitization of acoustic startle. Behavioral neuroscience 114:262-272.

Greenberg PE, Sisitsky T, Kessler RC, Finkelstein SN, Berndt ER, Davidson JR, Ballenger JC, Fyer AJ (1999) The economic burden of anxiety disorders in the 1990s. The Journal of clinical psychiatry 60:427-435.

Greenwald MK, Bradley MM, Cuthbert BN, Lang PJ (1998) Startle potentiation: shock sensitization, aversive learning, and affective picture modulation. Behavioral neuroscience 112:1069-1079.

Griebel G, Simiand J, Steinberg R, Jung M, Gully D, Roger P, Geslin M, Scatton B, Maffrand JP, Soubrie P (2002) 4-(2-Chloro-4-methoxy-5-methylphenyl)-N-[(1S)-2-cyclopropyl-1-(3-fluoro-4- methylphenyl)ethyl]5-methyl-N-(2-propynyl)-1, 3-thiazol-2-amine hydrochloride (SSR125543A), a potent and selective corticotrophin-releasing factor(1) receptor antagonist. II. Characterization in rodent models of stress-related disorders. The Journal of pharmacology and experimental therapeutics 301:333-345.

Grilli M, Neri E, Zappettini S, Massa F, Bisio A, Romussi G, Marchi M, Pittaluga A (2009) Salvinorin A exerts opposite presynaptic controls on neurotransmitter exocytosis from mouse brain nerve terminals. Neuropharmacology 57:523-530.

Grillon C, Davis M (1997) Fear-potentiated startle conditioning in humans: explicit and contextual cue conditioning following paired versus unpaired training. Psychophysiology 34:451-458.

Grillon C, Baas J (2003) A review of the modulation of the startle reflex by affective states and its application in psychiatry. Clinical neurophysiology : official journal of the International Federation of Clinical Neurophysiology 114:1557-1579.

Gross RA, Moises HC, Uhler MD, Macdonald RL (1990) Dynorphin A and cAMP-dependent protein kinase independently regulate neuronal calcium currents. Proceedings of the National Academy of Sciences of the United States of America 87:7025-7029.

Guarraci FA, Kapp BS (1999) An electrophysiological characterization of ventral tegmental area dopaminergic neurons during differential pavlovian fear conditioning in the awake rabbit. Behavioural brain research 99:169-179.

Gurvits TV, Shenton ME, Hokama H, Ohta H, Lasko NB, Gilbertson MW, Orr SP, Kikinis R, Jolesz FA, McCarley RW, Pitman RK (1996) Magnetic resonance imaging study of hippocampal volume in chronic, combat-related posttraumatic stress disorder. Biological psychiatry 40:1091-1099.

Habib KE, Weld KP, Rice KC, Pushkas J, Champoux M, Listwak S, Webster EL, Atkinson AJ, Schulkin J, Contoreggi C, Chrousos GP, McCann SM, Suomi SJ, Higley JD, Gold PW (2000) Oral administration of a corticotropin-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates. Proceedings of the National Academy of Sciences of the United States of America 97:6079-6084.

Halasy K, Racz B, Maderspach K (2000) Kappa opioid receptors are expressed by interneurons in the CA1 area of the rat hippocampus: a correlated light and electron microscopic immunocytochemical study. Journal of chemical neuroanatomy 19:233-241.

Hall CS (1934) Emotional behavior in the rat I. defacation and urination as measures of individual differences in emotionality. J Comp Psychol 18:385-403.

Hanson GR, Merchant KM, Letter AA, Bush L, Gibb JW (1988) Characterization of methamphetamine effects on the striatal-nigral dynorphin system. European journal of pharmacology 155:11-18.

Harrison AA, Everitt BJ, Robbins TW (1997) Central 5-HT depletion enhances impulsive responding without affecting the accuracy of attentional performance: interactions with dopaminergic mechanisms. Psychopharmacology 133:329-342.

Hasue RH, Shammah-Lagnado SJ (2002) Origin of the dopaminergic innervation of the central extended amygdala and accumbens shell: a combined retrograde tracing and immunohistochemical study in the rat. The Journal of comparative neurology 454:15-33.

Hauger RL, Risbrough V, Oakley RH, Olivares-Reyes JA, Dautzenberg FM (2009) Role of CRF receptor signaling in stress vulnerability, anxiety, and depression. Annals of the New York Academy of Sciences 1179:120-143.

Heidbreder CA, Goldberg SR, Shippenberg TS (1993) The kappa-opioid receptor agonist U-69593 attenuates cocaine-induced behavioral sensitization in the rat. Brain research 616:335-338.

Heidbreder CA, Babovic-Vuksanovic D, Shoaib M, Shippenberg TS (1995) Development of behavioral sensitization to cocaine: influence of kappa opioid receptor agonists. The Journal of pharmacology and experimental therapeutics 275:150-163.

Heim C, Nemeroff CB (1999) The impact of early adverse experiences on brain systems involved in the pathophysiology of anxiety and affective disorders. Biological psychiatry 46:1509-1522.

Heimer L, Alheid GF, de Olmos JS, Groenewegen HJ, Haber SN, Harlan RE, Zahm DS (1997) The accumbens: beyond the core-shell dichotomy. The Journal of neuropsychiatry and clinical neurosciences 9:354-381.

Henry DJ, Grandy DK, Lester HA, Davidson N, Chavkin C (1995) Kappa-opioid receptors couple to inwardly rectifying potassium channels when coexpressed by Xenopus oocytes. Molecular pharmacology 47:551-557.

Herman JP, Ostrander MM, Mueller NK, Figueiredo H (2005) Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. Progress in neuro-psychopharmacology & biological psychiatry 29:1201-1213.

Herman JP, Schafer MK, Young EA, Thompson R, Douglass J, Akil H, Watson SJ (1989) Evidence for hippocampal regulation of neuroendocrine neurons of the hypothalamo-pituitary-adrenocortical axis. The Journal of neuroscience : the official journal of the Society for Neuroscience 9:3072-3082.

Heuser I, Bissette G, Dettling M, Schweiger U, Gotthardt U, Schmider J, Lammers CH, Nemeroff CB, Holsboer F (1998) Cerebrospinal fluid concentrations of corticotropin-releasing hormone, vasopressin, and somatostatin in depressed patients and healthy controls: response to amitriptyline treatment. Depression and anxiety 8:71-79.

Hijzen TH, Houtzager SW, Joordens RJ, Olivier B, Slangen JL (1995) Predictive validity of the potentiated startle response as a behavioral model for anxiolytic drugs. Psychopharmacology 118:150-154.

Hitchcock J, Davis M (1986) Lesions of the amygdala, but not of the cerebellum or red nucleus, block conditioned fear as measured with the potentiated startle paradigm. Behavioral neuroscience 100:11-22.

Hitchcock JM, Davis M (1991) Efferent pathway of the amygdala involved in conditioned fear as measured with the fear-potentiated startle paradigm. Behavioral neuroscience 105:826-842.

Hitchcock JM, Sananes CB, Davis M (1989) Sensitization of the startle reflex by footshock: blockade by lesions of the central nucleus of the amygdala or its efferent pathway to the brainstem. Behavioral neuroscience 103:509-518.

Hjelmstad GO, Fields HL (2003) Kappa opioid receptor activation in the nucleus accumbens inhibits glutamate and GABA release through different mechanisms. Journal of neurophysiology 89:2389-2395.

Ho SP, Takahashi LK, Livanov V, Spencer K, Lesher T, Maciag C, Smith MA, Rohrbach KW, Hartig PR, Arneric SP (2001) Attenuation of fear conditioning by antisense inhibition of brain corticotropin releasing factor-2 receptor. Brain research Molecular brain research 89:29-40.

Holt W, Maren S (1999) Muscimol inactivation of the dorsal hippocampus impairs contextual retrieval of fear memory. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:9054-9062.

Horger BA, Iyasere CA, Berhow MT, Messer CJ, Nestler EJ, Taylor JR (1999) Enhancement of locomotor activity and conditioned reward to cocaine by brain-derived neurotrophic factor. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:4110-4122.

Horvitz JC (2000) Mesolimbocortical and nigrostriatal dopamine responses to salient non-reward events. Neuroscience 96:651-656.

Hurd YL (1996) Differential messenger RNA expression of prodynorphin and proenkephalin in the human brain. Neuroscience 72:767-783.

Imaki T, Shibasaki T, Hotta M, Demura H (1993) Intracerebroventricular administration of corticotropin-releasing factor induces c-fos mRNA expression in brain regions related to stress responses: comparison with pattern of c-fos mRNA induction after stress. Brain research 616:114-125.

Imperato A, Cabib S, Puglisi-Allegra S (1993) Repeated stressful experiences differently affect the time-dependent responses of the mesolimbic dopamine system to the stressor. Brain research 601:333-336.

Imperato A, Angelucci L, Casolini P, Zocchi A, Puglisi-Allegra S (1992) Repeated stressful experiences differently affect limbic dopamine release during and following stress. Brain research 577:194-199.

Inagaki S, Kawai Y, Matsuzaki T, Shiosaka S, Tohyama M (1983) Precise terminal fields of the descending somatostatinergic neuron system from the amygdaloid complex of the rat. Journal fur Hirnforschung 24:345-356.

Inan S, Cowan A (2004) Kappa opioid agonists suppress chloroquine-induced scratching in mice. European journal of pharmacology 502:233-237.

Inoue T, Tsuchiya K, Koyama T (1996) Effects of typical and atypical antipsychotic drugs on freezing behavior induced by conditioned fear. Pharmacology, biochemistry, and behavior 55:195-201.

Iwamoto ET (1985) Place-conditioning properties of mu, kappa, and sigma opioid agonists. Alcohol and drug research 6:327-339.

Jackson KJ, Carroll FI, Negus SS, Damaj MI (2010) Effect of the selective kappa-opioid receptor antagonist JDTic on nicotine antinociception, reward, and withdrawal in the mouse. Psychopharmacology 210:285-294.

James IF, Fischli W, Goldstein A (1984) Opioid receptor selectivity of dynorphin gene products. The Journal of pharmacology and experimental therapeutics 228:88-93.

Janssen PA, Niemegeers CJ, Dony JG (1963) The inhibitory effect of fentanyl and other morphine-like analgesics on the warm water induced tail withdrawl reflex in rats. Arzneimittel-Forschung 13:502-507.

Jones DN, Holtzman SG (1992) Long term kappa-opioid receptor blockade following nor-binaltorphimine. European journal of pharmacology 215:345-348.

Kalin NH (1990) Behavioral and endocrine studies of corticotropin-releasing hormone in primates. In: Corticotropin-Releasing Factor: Band and Clinical Studies of a Neuropeptide (De Souza EB, Nemeroff CB, eds), pp 275-289. Boca Raton: CRC Press.

Kam AY, Chan AS, Wong YH (2004) Kappa-opioid receptor signals through Src and focal adhesion kinase to stimulate c-Jun N-terminal kinases in transfected COS-7 cells and human monocytic THP-1 cells. The Journal of pharmacology and experimental therapeutics 310:301-310.

Karlsson KA, Blumberg MS (2004) Temperature-induced reciprocal activation of hippocampal field activity. Journal of neurophysiology 91:583-588.

Karolyi IJ, Burrows HL, Ramesh TM, Nakajima M, Lesh JS, Seong E, Camper SA, Seasholtz AF (1999) Altered anxiety and weight gain in corticotropin-releasing hormone-binding protein-deficient mice. Proceedings of the National Academy of Sciences of the United States of America 96:11595-11600.

Kasckow JW, Baker D, Geracioti TD, Jr. (2001) Corticotropin-releasing hormone in depression and post-traumatic stress disorder. Peptides 22:845-851.

Kaufman J, Charney D (2000) Comorbidity of mood and anxiety disorders. Depression and anxiety 12 Suppl 1:69-76.

Keane TM, Marshall AD, Taft CT (2006) Posttraumatic stress disorder: etiology, epidemiology, and treatment outcome. Annual review of clinical psychology 2:161-197.

Keay KA, Bandler R (2001) Parallel circuits mediating distinct emotional coping reactions to different types of stress. Neuroscience and biobehavioral reviews 25:669-678.

Keeney AJ, Hogg S (1999) Behavioural consequences of repeated social defeat in the mouse: preliminary evaluation of a potential animal model of depression. Behavioural pharmacology 10:753-764.

Keller MC, Neale MC, Kendler KS (2007) Association of different adverse life events with distinct patterns of depressive symptoms. The American journal of psychiatry 164:1521-1529; quiz 1622.

Kendler KS, Karkowski LM, Prescott CA (1999) Causal relationship between stressful life events and the onset of major depression. The American journal of psychiatry 156:837-841.

Kessler RC (1997) The effects of stressful life events on depression. Annual review of psychology 48:191-214.

Kessler RC (2000) The epidemiology of pure and comorbid generalized anxiety disorder: a review and evaluation of recent research. Acta psychiatrica Scandinavica Supplementum:7-13.

Kessler RC, Ruscio AM, Shear K, Wittchen HU (2010) Epidemiology of anxiety disorders. Current topics in behavioral neurosciences 2:21-35.

Kessler RC, Chiu WT, Demler O, Merikangas KR, Walters EE (2005a) Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Archives of general psychiatry 62:617-627.

Kessler RC, Berglund P, Demler O, Jin R, Merikangas KR, Walters EE (2005b) Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Archives of general psychiatry 62:593-602.

Kessler RC, Berglund P, Demler O, Jin R, Koretz D, Merikangas KR, Rush AJ, Walters EE, Wang PS (2003) The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). JAMA 289:3095-3105.

Khachaturian H, Watson SJ, Lewis ME, Coy D, Goldstein A, Akil H (1982) Dynorphin immunocytochemistry in the rat central nervous system. Peptides 3:941-954.

Kim JJ, Fanselow MS (1992) Modality-specific retrograde amnesia of fear. Science 256:675-677.

Kirby LG, Rice KC, Valentino RJ (2000) Effects of corticotropin-releasing factor on neuronal activity in the serotonergic dorsal raphe nucleus. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 22:148-162.

Kishimoto T, Radulovic J, Radulovic M, Lin CR, Schrick C, Hooshmand F, Hermanson O, Rosenfeld MG, Spiess J (2000) Deletion of crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. Nature genetics 24:415-419.

Knoll AT, Carlezon WA, Jr. (2010) Dynorphin, stress, and depression. Brain research 1314:56-73.

Knoll AT, Meloni EG, Thomas JB, Carroll FI, Carlezon WA, Jr. (2007) Anxiolytic-like effects of kappa-opioid receptor antagonists in models of unlearned and learned fear in rats. The Journal of pharmacology and experimental therapeutics 323:838-845.

Knoll AT, Muschamp JW, Sillivan SE, Ferguson D, Dietz DM, Meloni EG, Carroll FI, Nestler EJ, Konradi C, Carlezon WA, Jr. (2011) Kappa opioid receptor signaling in the basolateral amygdala regulates conditioned fear and anxiety in rats. Biological psychiatry 70:425-433.

Knott VJ, Millar AM, McIntosh JF, Shah DK, Fisher DJ, Blais CM, Ilivitsky V, Horn E (2011) Separate and combined effects of low dose ketamine and nicotine on behavioural and neural correlates of sustained attention. Biological psychology 88:83-93.

Koff JM, Shuster L, Miller LG (1994) Chronic cocaine administration is associated with behavioral sensitization and time-dependent changes in striatal dopamine transporter binding. The Journal of pharmacology and experimental therapeutics 268:277-282.

Konkoy CS, Childers SR (1989) Dynorphin-selective inhibition of adenylyl cyclase in guinea pig cerebellum membranes. Molecular pharmacology 36:627-633.

Konkoy CS, Childers SR (1993) Relationship between kappa 1 opioid receptor binding and inhibition of adenylyl cyclase in guinea pig brain membranes. Biochemical pharmacology 45:207-216.

Koob G, Kreek MJ (2007) Stress, dysregulation of drug reward pathways, and the transition to drug dependence. The American journal of psychiatry 164:1149-1159.

Koob GF (1999) Corticotropin-releasing factor, norepinephrine, and stress. Biological psychiatry 46:1167-1180.

Koob GF, Le Moal M (2008) Addiction and the brain antireward system. Annual review of psychology 59:29-53.

Koob GF, Heinrichs SC, Pich EM, Menzaghi F, Baldwin H, Miczek K, Britton KT (1993) The role of corticotropin-releasing factor in behavioural responses to stress. Ciba Found Symp 172:277-289; discussion 290-275.

Kotti T, Tapiola T, Riekkinen PJ, Sr., Miettinen R (1996) The calretinin-containing mossy cells survive excitotoxic insult in the gerbil dentate gyrus. Comparison of excitotoxicity-induced neuropathological changes in the gerbil and rat. The European journal of neuroscience 8:2371-2378.

Kovacs KJ (1998) c-Fos as a transcription factor: a stressful (re)view from a functional map. Neurochemistry international 33:287-297.

Krettek JE, Price JL (1978) A description of the amygdaloid complex in the rat and cat with observations on intra-amygdaloid axonal connections. The Journal of comparative neurology 178:255-280.

Krishnan V, Han MH, Mazei-Robison M, Iniguez SD, Ables JL, Vialou V, Berton O, Ghose S, Covington HE, 3rd, Wiley MD, Henderson RP, Neve RL, Eisch AJ, Tamminga CA, Russo SJ, Bolanos CA, Nestler EJ (2008) AKT signaling within the ventral tegmental area regulates cellular and behavioral responses to stressful stimuli. Biological psychiatry 64:691-700.

Kwon MS, Seo YJ, Shim EJ, Lee JK, Jang JE, Park SH, Jung JS, Suh HW (2008) The differential effects of emotional or physical stress on pain behaviors or on c-Fos immunoreactivity in paraventricular nucleus or arcuate nucleus. Brain research 1190:122-131.

La Regina A, Petrillo P, Sbacchi M, Tavani A (1988) Interaction of U-69,593 with mu-, alpha- and kappa-opioid binding sites and its analgesic and intestinal effects in rats. Life sciences 42:293-301.

Lammel S, Ion DI, Roeper J, Malenka RC (2011) Projection-specific modulation of dopamine neuron synapses by aversive and rewarding stimuli. Neuron 70:855-862.

Land BB, Bruchas MR, Lemos JC, Xu M, Melief EJ, Chavkin C (2008) The dysphoric component of stress is encoded by activation of the dynorphin kappa-opioid system. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:407-414.

Land BB, Bruchas MR, Schattauer S, Giardino WJ, Aita M, Messinger D, Hnasko TS, Palmiter RD, Chavkin C (2009) Activation of the kappa opioid receptor in the dorsal raphe nucleus mediates the aversive effects of stress and reinstates drug seeking. Proceedings of the National Academy of Sciences of the United States of America 106:19168-19173.

Lang PJ, Bradley MM, Cuthbert BN (1990) Emotion, attention, and the startle reflex. Psychological review 97:377-395.

Law PY, Wong YH, Loh HH (2000) Molecular mechanisms and regulation of opioid receptor signaling. Annual review of pharmacology and toxicology 40:389-430.

Lawrence DM, Bidlack JM (1993) The kappa opioid receptor expressed on the mouse R1.1 thymoma cell line is coupled to adenylyl cyclase through a pertussis toxin-sensitive guanine nucleotide-binding regulatory protein. The Journal of pharmacology and experimental therapeutics 266:1678-1683.

Le AD, Harding S, Juzytsch W, Fletcher PJ, Shaham Y (2002) The role of corticotropin-releasing factor in the median raphe nucleus in relapse to alcohol. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:7844-7849.

Leaton RN (1981) Habituation of startle response, lick suppression, and exploratory behavior in rats with hippocampal lesions. Journal of comparative and physiological psychology 95:813-826.

LeDoux JE (2000) Emotion circuits in the brain. Annual review of neuroscience 23:155-184.

Lee Y, Davis M (1997) Role of the hippocampus, the bed nucleus of the stria terminalis, and the amygdala in the excitatory effect of corticotropin-releasing hormone on the acoustic startle reflex. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:6434-6446.

Lee Y, Schulkin J, Davis M (1994) Effect of corticosterone on the enhancement of the acoustic startle reflex by corticotropin releasing factor (CRF). Brain research 666:93-98.

Lemos JC, Roth CA, Messinger DI, Gill HK, Phillips PE, Chavkin C (2012a) Repeated stress dysregulates kappa-opioid receptor signaling in the dorsal raphe through a p38alpha MAPK-dependent mechanism. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:12325-12336.

Lemos JC, Wanat MJ, Smith JS, Reyes BA, Hollon NG, Van Bockstaele EJ, Chavkin C, Phillips PE (2012b) Severe stress switches CRF action in the nucleus accumbens from appetitive to aversive. Nature 490:402-406.

Lett BT (1989) Repeated exposures intensify rather than diminish the rewarding effects of amphetamine, morphine, and cocaine. Psychopharmacology 98:357-362.

Lewis JW, Cannon JT, Liebeskind JC (1980) Opioid and nonopioid mechanisms of stress analgesia. Science 208:623-625.

Lewis JW, Terman GW, Watkins LR, Mayer DJ, Liebeskind JC (1983) Opioid and non-opioid mechanisms of footshock-induced analgesia: role of the spinal dorsolateral funiculus. Brain research 267:139-144.

Li C, Pleil KE, Stamatakis AM, Busan S, Vong L, Lowell BB, Stuber GD, Kash TL (2012) Presynaptic inhibition of gamma-aminobutyric acid release in the bed nucleus of the stria terminalis by kappa opioid receptor signaling. Biological psychiatry 71:725-732.

Li HY, Sawchenko PE (1998) Hypothalamic effector neurons and extended circuitries activated in "neurogenic" stress: a comparison of footshock effects exerted acutely, chronically, and in animals with controlled glucocorticoid levels. The Journal of comparative neurology 393:244-266.

Liang KC, Melia KR, Miserendino MJ, Falls WA, Campeau S, Davis M (1992) Corticotropin-releasing factor: long-lasting facilitation of the acoustic startle reflex. The Journal of neuroscience : the official journal of the Society for Neuroscience 12:2303-2312.

Liebsch G, Landgraf R, Engelmann M, Lorscher P, Holsboer F (1999) Differential behavioural effects of chronic infusion of CRH 1 and CRH 2 receptor antisense oligonucleotides into the rat brain. Journal of psychiatric research 33:153-163.

Lin S, Boey D, Lee N, Schwarzer C, Sainsbury A, Herzog H (2006) Distribution of prodynorphin mRNA and its interaction with the NPY system in the mouse brain. Neuropeptides 40:115-123.

Liu X, Ramirez S, Pang PT, Puryear CB, Govindarajan A, Deisseroth K, Tonegawa S (2012) Optogenetic stimulation of a hippocampal engram activates fear memory recall. Nature 484:381-385.

Lopez JF, Akil H, Watson SJ (1999) Neural circuits mediating stress. Biological psychiatry 46:1461-1471.

Lowenstein DH, Thomas MJ, Smith DH, McIntosh TK (1992) Selective vulnerability of dentate hilar neurons following traumatic brain injury: a potential mechanistic link between head trauma and disorders of the hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience 12:4846-4853.

Lowry CA, Johnson PL, Hay-Schmidt A, Mikkelsen J, Shekhar A (2005) Modulation of anxiety circuits by serotonergic systems. Stress 8:233-246.

Mague SD, Pliakas AM, Todtenkopf MS, Tomasiewicz HC, Zhang Y, Stevens WC, Jr., Jones RM, Portoghese PS, Carlezon WA, Jr. (2003) Antidepressant-like effects of kappa-opioid receptor antagonists in the forced swim test in rats. The Journal of pharmacology and experimental therapeutics 305:323-330.

Mahan AL, Ressler KJ (2012) Fear conditioning, synaptic plasticity and the amygdala: implications for posttraumatic stress disorder. Trends in neurosciences 35:24-35.

Maisonneuve IM, Archer S, Glick SD (1994) U50,488, a kappa opioid receptor agonist, attenuates cocaine-induced increases in extracellular dopamine in the nucleus accumbens of rats. Neuroscience letters 181:57-60.

Majzoub J (2006) Corticotropin-releasing hormone physiology. Eur J Endocrinol 155:S71-S76.

Mansour A, Fox CA, Akil H, Watson SJ (1995) Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. Trends in neurosciences 18:22-29.

Mansour A, Khachaturian H, Lewis ME, Akil H, Watson SJ (1987) Autoradiographic differentiation of mu, delta, and kappa opioid receptors in the rat forebrain and midbrain. The Journal of neuroscience : the official journal of the Society for Neuroscience 7:2445-2464.

Mansour A, Khachaturian H, Lewis ME, Akil H, Watson SJ (1988) Anatomy of CNS opioid receptors. Trends in neurosciences 11:308-314.

Marchant NJ, Densmore VS, Osborne PB (2007) Coexpression of prodynorphin and corticotrophin-releasing hormone in the rat central amygdala: evidence of two distinct endogenous opioid systems in the lateral division. The Journal of comparative neurology 504:702-715.

Marco EM, Adriani W, Llorente R, Laviola G, Viveros MP (2009) Detrimental psychophysiological effects of early maternal deprivation in adolescent and adult rodents: altered responses to cannabinoid exposure. Neuroscience and biobehavioral reviews 33:498-507.

Maren S, Quirk GJ (2004) Neuronal signalling of fear memory. Nature reviews Neuroscience 5:844-852.

Margolis EB, Hjelmstad GO, Bonci A, Fields HL (2003) Kappa-opioid agonists directly inhibit midbrain dopaminergic neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:9981-9986.

Margolis EB, Hjelmstad GO, Bonci A, Fields HL (2005) Both kappa and mu opioid agonists inhibit glutamatergic input to ventral tegmental area neurons. Journal of neurophysiology 93:3086-3093.

Margolis EB, Lock H, Hjelmstad GO, Fields HL (2006a) The ventral tegmental area revisited: is there an electrophysiological marker for dopaminergic neurons? The Journal of physiology 577:907-924.

Margolis EB, Mitchell JM, Ishikawa J, Hjelmstad GO, Fields HL (2008) Midbrain dopamine neurons: projection target determines action potential duration and dopamine D(2) receptor inhibition. The Journal of neuroscience : the official journal of the Society for Neuroscience 28:8908-8913.

Margolis EB, Lock H, Chefer VI, Shippenberg TS, Hjelmstad GO, Fields HL (2006b) Kappa opioids selectively control dopaminergic neurons projecting to the prefrontal cortex. Proceedings of the National Academy of Sciences of the United States of America 103:2938-2942.

Marrosu F, Pinna A, Fadda P, Fratta W, Morelli M (1996) C-Fos expression as a molecular marker in corticotropin-releasing factor-induced seizures. Synapse 24:297-304.

McCullough LD, Salamone JD (1992) Anxiogenic drugs beta-CCE and FG 7142 increase extracellular dopamine levels in nucleus accumbens. Psychopharmacology 109:379-382.

McDermott CM, Schrader LA (2011) Activation of kappa opioid receptors increases intrinsic excitability of dentate gyrus granule cells. The Journal of physiology 589:3517-3532.

McDonald AJ, Mascagni F, Guo L (1996) Projections of the medial and lateral prefrontal cortices to the amygdala: a Phaseolus vulgaris leucoagglutinin study in the rat. Neuroscience 71:55-75.

McEwen BS, Magarinos AM (2001) Stress and hippocampal plasticity: implications for the pathophysiology of affective disorders. Human psychopharmacology 16:S7-S19.

McLaughlin JP, Marton-Popovici M, Chavkin C (2003a) Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:5674-5683.

McLaughlin JP, Xu M, Mackie K, Chavkin C (2003b) Phosphorylation of a carboxyl-terminal serine within the kappa-opioid receptor produces desensitization and internalization. The Journal of biological chemistry 278:34631-34640.

McLaughlin JP, Land BB, Li S, Pintar JE, Chavkin C (2006a) Prior activation of kappa opioid receptors by U50,488 mimics repeated forced swim stress to potentiate cocaine place preference conditioning. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 31:787-794.

McLaughlin JP, Li S, Valdez J, Chavkin TA, Chavkin C (2006b) Social defeat stress-induced behavioral responses are mediated by the endogenous kappa opioid system. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 31:1241-1248.

McLaughlin JP, Myers LC, Zarek PE, Caron MG, Lefkowitz RJ, Czyzyk TA, Pintar JE, Chavkin C (2004) Prolonged kappa opioid receptor phosphorylation mediated by G-protein receptor kinase underlies sustained analgesic tolerance. The Journal of biological chemistry 279:1810-1818.

McNish KA, Gewirtz JC, Davis M (1997) Evidence of contextual fear after lesions of the hippocampus: a disruption of freezing but not fear-potentiated startle. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:9353-9360.

Melief EJ, Miyatake M, Bruchas MR, Chavkin C (2010) Ligand-directed c-Jun N-terminal kinase activation disrupts opioid receptor signaling. Proceedings of the National Academy of Sciences of the United States of America 107:11608-11613.

Melief EJ, Miyatake M, Carroll FI, Beguin C, Carlezon WA, Jr., Cohen BM, Grimwood S, Mitch CH, Rorick-Kehn L, Chavkin C (2011) Duration of action of a broad range of selective kappa-opioid receptor antagonists is positively correlated with c-Jun N-terminal kinase-1 activation. Molecular pharmacology 80:920-929.

Meloni EG, Gerety LP, Knoll AT, Cohen BM, Carlezon WA, Jr. (2006) Behavioral and anatomical interactions between dopamine and corticotropin-releasing factor in the rat. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:3855-3863.

Menard J, Treit D (2001) The anxiolytic effects of intra-hippocampal midazolam are antagonized by intra-septal L-glutamate. Brain research 888:163-166.

Menendez L, Andres-Trelles F, Hidalgo A, Baamonde A (1993a) Involvement of spinal kappa opioid receptors in a type of footshock induced analgesia in mice. Brain research 611:264-271.

Menendez L, Andres-Trelles F, Hidalgo A, Baamonde A (1993b) Opioid footshock-induced analgesia in mice acutely falls by stress prolongation. Physiology & behavior 53:1115-1119.

Meng F, Xie GX, Thompson RC, Mansour A, Goldstein A, Watson SJ, Akil H (1993) Cloning and pharmacological characterization of a rat kappa opioid receptor. Proceedings of the National Academy of Sciences of the United States of America 90:9954-9958.

Merchenthaler I (1984) Corticotropin releasing factor (CRF)-like immunoreactivity in the rat central nervous system. Extrahypothalamic distribution. Peptides 5 Suppl 1:53-69.

Merchenthaler I, Maderdrut JL, Cianchetta P, Shughrue P, Bronstein D (1997) In situ hybridization histochemical localization of prodynorphin messenger RNA in the central nervous system of the rat. The Journal of comparative neurology 384:211-232.

Mickley GA, Ferguson JL (1989) Enhanced acoustic startle responding in rats with radiation-induced hippocampal granule cell hypoplasia. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 75:28-34.

Miczek KA, Covington HE, 3rd, Nikulina EM, Jr., Hammer RP (2004) Aggression and defeat: persistent effects on cocaine self-administration and gene expression in peptidergic and aminergic mesocorticolimbic circuits. Neuroscience and biobehavioral reviews 27:787-802.

Millan MA, Jacobowitz DM, Hauger RL, Catt KJ, Aguilera G (1986) Distribution of corticotropin-releasing factor receptors in primate brain. Proceedings of the National Academy of Sciences of the United States of America 83:1921-1925.

Millhouse OE (1986) The intercalated cells of the amygdala. The Journal of comparative neurology 247:246-271.

Misawa H, Ueda H, Satoh M (1990) Kappa-opioid agonist inhibits phospholipase C, possibly via an inhibition of G-protein activity. Neuroscience letters 112:324-327.

Montgomery EB, Jr., Gale JT (2008) Mechanisms of action of deep brain stimulation(DBS). Neuroscience and biobehavioral reviews 32:388-407.

Moreau JL, Jenck F, Martin JR, Mortas P, Haefely WE (1992) Antidepressant treatment prevents chronic unpredictable mild stress-induced anhedonia as assessed by ventral tegmentum self-stimulation behavior in rats. European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology 2:43-49.

Morgan CA, 3rd, Southwick SM, Grillon C, Davis M, Krystal JH, Charney DS (1993) Yohimbine-facilitated acoustic startle reflex in humans. Psychopharmacology 110:342-346.

Morgan JI, Curran T (1991) Stimulus-transcription coupling in the nervous system: involvement of the inducible proto-oncogenes fos and jun. Annual review of neuroscience 14:421-451.

Morimoto M, Morita N, Ozawa H, Yokoyama K, Kawata M (1996) Distribution of glucocorticoid receptor immunoreactivity and mRNA in the rat brain: an immunohistochemical and in situ hybridization study. Neuroscience research 26:235-269.

Morris BJ, Haarmann I, Kempter B, Hollt V, Herz A (1986) Localization of prodynorphin messenger rna in rat brain by in situ hybridization using a synthetic oligonucleotide probe. Neuroscience letters 69:104-108.

Mucha M, Skrzypiec AE, Schiavon E, Attwood BK, Kucerova E, Pawlak R (2011) Lipocalin-2 controls neuronal excitability and anxiety by regulating dendritic spine formation and maturation. Proceedings of the National Academy of Sciences of the United States of America 108:18436-18441.

Munck A, Guyre PM, Holbrook NJ (1984) Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocrine reviews 5:25-44.

Munro LJ, Kokkinidis L (1997) Infusion of quinpirole and muscimol into the ventral tegmental area inhibits fear-potentiated startle: implications for the role of dopamine in fear expression. Brain research 746:231-238.

Munro TA, Berry LM, Van't Veer A, Beguin C, Carroll FI, Zhao Z, Carlezon WA, Jr., Cohen BM (2012) Long-acting kappa opioid antagonists nor-BNI, GNTI and JDTic: pharmacokinetics in mice and lipophilicity. BMC pharmacology 12:5.

Muschamp JW, Van't Veer A, Carlezon WA, Jr. (2011a) Tracking down the molecular substrates of stress: new roles for p38alpha MAPK and kappa-opioid receptors. Neuron 71:383-385.

Muschamp JW, Nemeth CL, Robison AJ, Nestler EJ, Carlezon WA, Jr. (2012) DeltaFosB enhances the rewarding effects of cocaine while reducing the pro-depressive effects of the kappa-opioid receptor agonist U50488. Biological psychiatry 71:44-50.

Muschamp JW, Van't Veer A, Parsegian A, Gallo MS, Chen M, Neve RL, Meloni EG, Carlezon WA, Jr. (2011b) Activation of CREB in the nucleus accumbens shell produces anhedonia and resistance to extinction of fear in rats. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:3095-3103.

Nabeshima T, Katoh A, Wada M, Kameyama T (1992) Stress-induced changes in brain Met-enkephalin, Leu-enkephalin and dynorphin concentrations. Life sciences 51:211-217.

Nader K, LeDoux JE (1999) Inhibition of the mesoamygdala dopaminergic pathway impairs the retrieval of conditioned fear associations. Behavioral neuroscience 113:891-901.

National Research Council (U.S.). Committee for the Update of the Guide for the Care and Use of Laboratory Animals., Institute for Laboratory Animal Research (U.S.), National Academies Press (U.S.) (2011) Guide for the care and use of laboratory animals, 8th Edition. Washington, D.C.: National Academies Press.

Negus SS (2004) Effects of the kappa opioid agonist U50,488 and the kappa opioid antagonist nor-binaltorphimine on choice between cocaine and food in rhesus monkeys. Psychopharmacology 176:204-213.

Negus SS, Mello NK, Portoghese PS, Lin CE (1997) Effects of kappa opioids on cocaine self-administration by rhesus monkeys. The Journal of pharmacology and experimental therapeutics 282:44-55.

Nemeroff CB (1992) New vistas in neuropeptide research in neuropsychiatry: focus on corticotropin-releasing factor. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 6:69-75.

Nemeroff CB, Widerlov E, Bissette G, Walleus H, Karlsson I, Eklund K, Kilts CD, Loosen PT, Vale W (1984) Elevated concentrations of CSF corticotropin-releasing factor-like immunoreactivity in depressed patients. Science 226:1342-1344.

Nemeth CL, Paine TA, Rittiner JE, Beguin C, Carroll FI, Roth BL, Cohen BM, Carlezon WA, Jr. (2010) Role of kappa-opioid receptors in the effects of salvinorin A and ketamine on attention in rats. Psychopharmacology 210:263-274.

Nestler EJ, Carlezon WA, Jr. (2006) The mesolimbic dopamine reward circuit in depression. Biological psychiatry 59:1151-1159.

Neumaier JF, Mailheau S, Chavkin C (1988) Opioid receptor-mediated responses in the dentate gyrus and CA1 region of the rat hippocampus. The Journal of pharmacology and experimental therapeutics 244:564-570.

Neves G, Cooke SF, Bliss TV (2008) Synaptic plasticity, memory and the hippocampus: a neural network approach to causality. Nature reviews Neuroscience 9:65-75.

Newton SS, Thome J, Wallace TL, Shirayama Y, Schlesinger L, Sakai N, Chen J, Neve R, Nestler EJ, Duman RS (2002) Inhibition of cAMP response element-binding protein or dynorphin in the nucleus accumbens produces an antidepressant-like effect. The Journal of neuroscience : the official journal of the Society for Neuroscience 22:10883-10890.

Nikolarakis KE, Almeida OF, Herz A (1986) Stimulation of hypothalamic beta-endorphin and dynorphin release by corticotropin-releasing factor (in vitro). Brain research 399:152-155.

Nikoshkov A, Hurd YL, Yakovleva T, Bazov I, Marinova Z, Cebers G, Pasikova N, Gharibyan A, Terenius L, Bakalkin G (2005) Prodynorphin transcripts and proteins differentially expressed and regulated in the adult human brain. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 19:1543-1545.

Nowak LG, Bullier J (1998) Axons, but not cell bodies, are activated by electrical stimulation in cortical gray matter. II. Evidence from selective inactivation of cell bodies

and axon initial segments. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 118:489-500.

O'Keefe JM, Nadel L (1978) The hippocampus as a cognitive map. Oxford New York: Clarendon Press ; Oxford University Press.

Ohmura Y, Yamaguchi T, Futami Y, Togashi H, Izumi T, Matsumoto M, Yoshida T, Yoshioka M (2009) Corticotropin releasing factor enhances attentional function as assessed by the five-choice serial reaction time task in rats. Behavioural brain research 198:429-433.

Owens MJ, Nemeroff CB (1991) Physiology and pharmacology of corticotropin-releasing factor. Pharmacological reviews 43:425-473.

Owens MJ, Vargas MA, Nemeroff CB (1993) The effects of alprazolam on corticotropin-releasing factor neurons in the rat brain: implications for a role for CRF in the pathogenesis of anxiety disorders. Journal of psychiatric research 27 Suppl 1:209-220.

Paine TA, Tomasiewicz HC, Zhang K, Carlezon WA, Jr. (2007) Sensitivity of the five-choice serial reaction time task to the effects of various psychotropic drugs in Sprague-Dawley rats. Biological psychiatry 62:687-693.

Pan ZZ (1998) mu-Opposing actions of the kappa-opioid receptor. Trends in pharmacological sciences 19:94-98.

Pare D, Quirk GJ, Ledoux JE (2004) New vistas on amygdala networks in conditioned fear. Journal of neurophysiology 92:1-9.

Parlato R, Rieker C, Turiault M, Tronche F, Schutz G (2006) Survival of DA neurons is independent of CREM upregulation in absence of CREB. Genesis 44:454-464.

Pascucci T, Ventura R, Latagliata EC, Cabib S, Puglisi-Allegra S (2007) The medial prefrontal cortex determines the accumbens dopamine response to stress through the opposing influences of norepinephrine and dopamine. Cerebral cortex 17:2796-2804.

Pasternak GW (1980) Multiple opiate receptors: [3H]ethylketocyclazocine receptor binding and ketocyclazocine analgesia. Proceedings of the National Academy of Sciences of the United States of America 77:3691-3694.

Paxinos G, Franklin KBJ (2001) The mouse brain in stereotaxic coordinates. San Diego: Academic Press; 2nd edition.

Peckys D, Landwehrmeyer GB (1999) Expression of mu, kappa, and delta opioid receptor messenger RNA in the human CNS: a 33P in situ hybridization study. Neuroscience 88:1093-1135.

Pelleymounter MA, Joppa M, Ling N, Foster AC (2002) Pharmacological evidence supporting a role for central corticotropin-releasing factor(2) receptors in behavioral, but not endocrine, response to environmental stress. The Journal of pharmacology and experimental therapeutics 302:145-152.

Petrovich GD, Swanson LW (1997) Projections from the lateral part of the central amygdalar nucleus to the postulated fear conditioning circuit. Brain research 763:247-254.

Pezze MA, Feldon J (2004) Mesolimbic dopaminergic pathways in fear conditioning. Progress in neurobiology 74:301-320.

Pezze MA, Feldon J, Murphy CA (2002) Increased conditioned fear response and altered balance of dopamine in the shell and core of the nucleus accumbens during amphetamine withdrawal. Neuropharmacology 42:633-643.

Pezze MA, Bast T, Feldon J (2003) Significance of dopamine transmission in the rat medial prefrontal cortex for conditioned fear. Cerebral cortex 13:371-380.

Pezzone MA, Lee WS, Hoffman GE, Rabin BS (1992) Induction of c-Fos immunoreactivity in the rat forebrain by conditioned and unconditioned aversive stimuli. Brain research 597:41-50.

Pfeiffer A, Brantl V, Herz A, Emrich HM (1986) Psychotomimesis mediated by kappa opiate receptors. Science 233:774-776.

Phillips RG, LeDoux JE (1992) Differential contribution of amygdala and hippocampus to cued and contextual fear conditioning. Behavioral neuroscience 106:274-285.

Phillipson OT (1979) The cytoarchitecture of the interfascicular nucleus and ventral tegmental area of Tsai in the rat. The Journal of comparative neurology 187:85-98.

Piazza PV, Le Moal M (1998) The role of stress in drug self-administration. Trends in pharmacological sciences 19:67-74.

Piazza PV, Deminiere JM, Le Moal M, Simon H (1989) Factors that predict individual vulnerability to amphetamine self-administration. Science 245:1511-1513.

Pickel VM, Chan J, Sesack SR (1993) Cellular substrates for interactions between dynorphin terminals and dopamine dendrites in rat ventral tegmental area and substantia nigra. Brain research 602:275-289.

Pine DS, Cohen P, Johnson JG, Brook JS (2002) Adolescent life events as predictors of adult depression. Journal of affective disorders 68:49-57.

Pitkanen A, Pikkarainen M, Nurminen N, Ylinen A (2000) Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. A review. Annals of the New York Academy of Sciences 911:369-391.

Platt JE, Stone EA (1982) Chronic restraint stress elicits a positive antidepressant response on the forced swim test. European journal of pharmacology 82:179-181.

Pliakas AM, Carlson RR, Neve RL, Konradi C, Nestler EJ, Carlezon WA, Jr. (2001) Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens. The Journal of neuroscience : the official journal of the Society for Neuroscience 21:7397-7403.

Porsolt RD, Le Pichon M, Jalfre M (1977a) Depression: a new animal model sensitive to antidepressant treatments. Nature 266:730-732.

Porsolt RD, Bertin A, Jalfre M (1977b) Behavioral despair in mice: a primary screening test for antidepressants. Archives internationales de pharmacodynamie et de therapie 229:327-336.

Price ML, Curtis AL, Kirby LG, Valentino RJ, Lucki I (1998) Effects of corticotropin-releasing factor on brain serotonergic activity. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 18:492-502.

Prut L, Belzung C (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. European journal of pharmacology 463:3-33.

Przewlocki R, Lason W, Hollt V, Silberring J, Herz A (1987) The influence of chronic stress on multiple opioid peptide systems in the rat: pronounced effects upon dynorphin in spinal cord. Brain research 413:213-219.

Quirk GJ, Mueller D (2008) Neural mechanisms of extinction learning and retrieval. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 33:56-72.

Quirk GJ, Likhtik E, Pelletier JG, Pare D (2003) Stimulation of medial prefrontal cortex decreases the responsiveness of central amygdala output neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:8800-8807.

Radke AK (2009) The role of the bed nucleus of the stria terminalis in learning to fear. The Journal of neuroscience : the official journal of the Society for Neuroscience 29:15351-15352.

Ratzliff A, Santhakumar V, Howard A, Soltesz I (2002) Mossy cells in epilepsy: rigor mortis or vigor mortis? Trends in neurosciences 25:140-144.

Ray WJ, Molnar C, Aikins D, Yamasaki A, Newman MG, Castonguay L, Borkovec TD (2009) Startle response in generalized anxiety disorder. Depression and anxiety 26:147-154.

Redila VA, Chavkin C (2008) Stress-induced reinstatement of cocaine seeking is mediated by the kappa opioid system. Psychopharmacology 200:59-70.

Refojo D, Schweizer M, Kuehne C, Ehrenberg S, Thoeringer C, Vogl AM, Dedic N, Schumacher M, von Wolff G, Avrabos C, Touma C, Engblom D, Schutz G, Nave KA, Eder M, Wotjak CT, Sillaber I, Holsboer F, Wurst W, Deussing JM (2011) Glutamatergic and dopaminergic neurons mediate anxiogenic and anxiolytic effects of CRHR1. Science 333:1903-1907.

Reul JM, de Kloet ER (1985) Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117:2505-2511.

Reul JM, Holsboer F (2002) Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Curr Opin Pharmacol 2:23-33.

Rezayat M, Roohbakhsh A, Zarrindast MR, Massoudi R, Djahanguiri B (2005) Cholecystokinin and GABA interaction in the dorsal hippocampus of rats in the elevated plus-maze test of anxiety. Physiology & behavior 84:775-782.

Richardson R (2000) Shock sensitization of startle: learned or unlearned fear? Behavioural brain research 110:109-117.

Risbrough VB, Geyer MA (2005) Anxiogenic treatments do not increase fear-potentiated startle in mice. Biological psychiatry 57:33-43.

Risbrough VB, Hauger RL, Roberts AL, Vale WW, Geyer MA (2004) Corticotropin-releasing factor receptors CRF1 and CRF2 exert both additive and opposing influences on defensive startle behavior. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:6545-6552.

Risbrough VB, Geyer MA, Hauger RL, Coste S, Stenzel-Poore M, Wurst W, Holsboer F (2009) CRF1 and CRF2 receptors are required for potentiated startle to contextual but not discrete cues. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 34:1494-1503.

Robbins TW (2002) The 5-choice serial reaction time task: behavioural pharmacology and functional neurochemistry. Psychopharmacology 163:362-380.

Robinson TE, Berridge KC (2000) The psychology and neurobiology of addiction: an incentive-sensitization view. Addiction 95 Suppl 2:S91-117.

Rogala B, Li Y, Li S, Chen X, Kirouac GJ (2012) Effects of a Post-Shock Injection of the Kappa Opioid Receptor Antagonist Norbinaltorphimine (norBNI) on Fear and Anxiety in Rats. PloS one 7:e49669.

Rogers RD, Baunez C, Everitt BJ, Robbins TW (2001) Lesions of the medial and lateral striatum in the rat produce differential deficits in attentional performance. Behavioral neuroscience 115:799-811.

Ronken E, Van Muiswinkel FL, Mulder AH, Schoffelmeer AN (1993) Opioid receptor-mediated inhibition of evoked catecholamine release from cultured neurons of rat ventral mesencephalon and locus coeruleus. European journal of pharmacology 230:349-355.

Rosen JB, Hitchcock JM, Sananes CB, Miserendino MJ, Davis M (1991) A direct projection from the central nucleus of the amygdala to the acoustic startle pathway: anterograde and retrograde tracing studies. Behavioral neuroscience 105:817-825.

Ross RT, Randich A (1984) Unconditioned stress-induced analgesia following exposure to brief footshock. Journal of experimental psychology Animal behavior processes 10:127-137.

Rossi NA, Reid LD (1976) Affective states associated with morphine injections. Physiological Psychology 4:269-274.

Rosvold HE, Mirsky AF, Sarason I, Bransome ED, Jr., Beck LH (1956) A continuous performance test of brain damage. Journal of consulting psychology 20:343-350.

Roth RH, Tam SY, Ida Y, Yang JX, Deutch AY (1988) Stress and the mesocorticolimbic dopamine systems. Annals of the New York Academy of Sciences 537:138-147.

Roybal K, Theobold D, Graham A, DiNieri JA, Russo SJ, Krishnan V, Chakravarty S, Peevey J, Oehrlein N, Birnbaum S, Vitaterna MH, Orsulak P, Takahashi JS, Nestler EJ, Carlezon WA, Jr., McClung CA (2007) Mania-like behavior induced by disruption of CLOCK. Proceedings of the National Academy of Sciences of the United States of America 104:6406-6411.

Rudy JW, Barrientos RM, O'Reilly RC (2002) Hippocampal formation supports conditioning to memory of a context. Behavioral neuroscience 116:530-538.

Rusin KI, Giovannucci DR, Stuenkel EL, Moises HC (1997) Kappa-opioid receptor activation modulates Ca2+ currents and secretion in isolated neuroendocrine nerve terminals. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:6565-6574.

Sah P, Faber ES, Lopez De Armentia M, Power J (2003) The amygdaloid complex: anatomy and physiology. Physiological reviews 83:803-834.

Sakanaka M, Shibasaki T, Lederis K (1986) Distribution and efferent projections of corticotropin-releasing factor-like immunoreactivity in the rat amygdaloid complex. Brain research 382:213-238.

Sakanaka M, Shibasaki T, Lederis K (1987) Corticotropin releasing factor-like immunoreactivity in the rat brain as revealed by a modified cobalt-glucose oxidase-diaminobenzidine method. The Journal of comparative neurology 260:256-298.

Salamone JD (1994) The involvement of nucleus accumbens dopamine in appetitive and aversive motivation. Behavioural brain research 61:117-133.

Sasaki H, Hanamoto A (2007) Shock sensitization and fear potentiation of auditory startle response in hamsters. Perceptual and motor skills 105:862-871.

Sautter FJ, Bissette G, Wiley J, Manguno-Mire G, Schoenbachler B, Myers L, Johnson JE, Cerbone A, Malaspina D (2003) Corticotropin-releasing factor in posttraumatic stress disorder (PTSD) with secondary psychotic symptoms, nonpsychotic PTSD, and healthy control subjects. Biological psychiatry 54:1382-1388.

Schindler AG, Li S, Chavkin C (2010) Behavioral stress may increase the rewarding valence of cocaine-associated cues through a dynorphin/kappa-opioid receptor-mediated mechanism without affecting associative learning or memory retrieval mechanisms. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 35:1932-1942.

Schmid A, Koch M, Schnitzler HU (1995) Conditioned pleasure attenuates the startle response in rats. Neurobiology of learning and memory 64:1-3.

Schwaber JS, Kapp BS, Higgins GA, Rapp PR (1982) Amygdaloid and basal forebrain direct connections with the nucleus of the solitary tract and the dorsal motor nucleus. The Journal of neuroscience : the official journal of the Society for Neuroscience 2:1424-1438.

Schwarzer C (2009) 30 years of dynorphins--new insights on their functions in neuropsychiatric diseases. Pharmacology & therapeutics 123:353-370.

Selye H (1956) The stress of life. New York,: McGraw-Hill.

Seress L, Ribak CE (1983) GABAergic cells in the dentate gyrus appear to be local circuit and projection neurons. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale 50:173-182.

Shapira NA, Okun MS, Wint D, Foote KD, Byars JA, Bowers D, Springer US, Lang PJ, Greenberg BD, Haber SN, Goodman WK (2006) Panic and fear induced by deep brain stimulation. Journal of neurology, neurosurgery, and psychiatry 77:410-412.

Shimada S, Kitayama S, Walther D, Uhl G (1992) Dopamine transporter mRNA: dense expression in ventral midbrain neurons. Brain research Molecular brain research 13:359-362.

Shin LM, Liberzon I (2010) The neurocircuitry of fear, stress, and anxiety disorders. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 35:169-191.

Shippenberg TS, Herz A (1987) Place preference conditioning reveals the involvement of D1-dopamine receptors in the motivational properties of mu- and kappa-opioid agonists. Brain research 436:169-172.

Shippenberg TS, Heidbreder C (1995) Sensitization to the conditioned rewarding effects of cocaine: pharmacological and temporal characteristics. The Journal of pharmacology and experimental therapeutics 273:808-815.

Shirayama Y, Ishida H, Iwata M, Hazama GI, Kawahara R, Duman RS (2004) Stress increases dynorphin immunoreactivity in limbic brain regions and dynorphin antagonism produces antidepressant-like effects. Journal of neurochemistry 90:1258-1268.

Si W, Aluisio L, Okamura N, Clark SD, Fraser I, Sutton SW, Bonaventure P, Reinscheid RK (2010) Neuropeptide S stimulates dopaminergic neurotransmission in the medial prefrontal cortex. Journal of neurochemistry 115:475-482.

Simmons ML, Chavkin C (1996) k-Opioid receptor activation of a dendrotoxin-sensitive potassium channel mediates presynaptic inhibition of mossy fiber neurotransmitter release. Molecular pharmacology 50:80-85.

Simonin F, Gaveriaux-Ruff C, Befort K, Matthes H, Lannes B, Micheletti G, Mattei MG, Charron G, Bloch B, Kieffer B (1995) kappa-Opioid receptor in humans: cDNA and genomic cloning, chromosomal assignment, functional expression, pharmacology, and expression pattern in the central nervous system. Proceedings of the National Academy of Sciences of the United States of America 92:7006-7010.

Simonin F, Valverde O, Smadja C, Slowe S, Kitchen I, Dierich A, Le Meur M, Roques BP, Maldonado R, Kieffer BL (1998) Disruption of the kappa-opioid receptor gene in

mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. The EMBO journal 17:886-897.

Sirinathsinghji DJ, Nikolarakis KE, Herz A (1989) Corticotropin-releasing factor stimulates the release of methionine-enkephalin and dynorphin from the neostriatum and globus pallidus of the rat: in vitro and in vivo studies. Brain research 490:276-291.

Sivam SP (1989) Cocaine selectively increases striatonigral dynorphin levels by a dopaminergic mechanism. The Journal of pharmacology and experimental therapeutics 250:818-824.

Skelton KH, Nemeroff CB, Knight DL, Owens MJ (2000) Chronic administration of the triazolobenzodiazepine alprazolam produces opposite effects on corticotropin-releasing factor and urocortin neuronal systems. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:1240-1248.

Slattery DA, Uschold N, Magoni M, Bar J, Popoli M, Neumann ID, Reber SO (2012) Behavioural consequences of two chronic psychosocial stress paradigms: Anxiety without depression. Psychoneuroendocrinology 37:702-714.

Smagin GN, Heinrichs SC, Dunn AJ (2001) The role of CRH in behavioral responses to stress. Peptides 22:713-724.

Smiley PL, Johnson M, Bush L, Gibb JW, Hanson GR (1990) Effects of cocaine on extrapyramidal and limbic dynorphin systems. The Journal of pharmacology and experimental therapeutics 253:938-943.

Smith GW, Aubry JM, Dellu F, Contarino A, Bilezikjian LM, Gold LH, Chen R, Marchuk Y, Hauser C, Bentley CA, Sawchenko PE, Koob GF, Vale W, Lee KF (1998) Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. Neuron 20:1093-1102.

Smith JS, Schindler AG, Martinelli E, Gustin RM, Bruchas MR, Chavkin C (2012) Stress-induced activation of the dynorphin/kappa-opioid receptor system in the amygdala potentiates nicotine conditioned place preference. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:1488-1495.

Smith MA, French AM (2002) Age-related differences in sensitivity to the antinociceptive effects of kappa opioids in adult male rats. Psychopharmacology 162:255-264.

Smith MA, Banerjee S, Gold PW, Glowa J (1992) Induction of c-fos mRNA in rat brain by conditioned and unconditioned stressors. Brain research 578:135-141.

Song ZH, Takemori AE (1992) Stimulation by corticotropin-releasing factor of the release of immunoreactive dynorphin A from mouse spinal cords in vitro. European journal of pharmacology 222:27-32.

Sorg BA, Kalivas PW (1991) Effects of cocaine and footshock stress on extracellular dopamine levels in the ventral striatum. Brain research 559:29-36.

Sorg BA, Kalivas PW (1993) Effects of cocaine and footshock stress on extracellular dopamine levels in the medial prefrontal cortex. Neuroscience 53:695-703.

Spanagel R, Herz A, Shippenberg TS (1992) Opposing tonically active endogenous opioid systems modulate the mesolimbic dopaminergic pathway. Proceedings of the National Academy of Sciences of the United States of America 89:2046-2050.

Sperling RE, Gomes SM, Sypek EI, Carey AN, McLaughlin JP (2010) Endogenous kappa-opioid mediation of stress-induced potentiation of ethanol-conditioned place preference and self-administration. Psychopharmacology 210:199-209.

Spiess J, Rivier J, Rivier C, Vale W (1981) Primary structure of corticotropin-releasing factor from ovine hypothalamus. Proceedings of the National Academy of Sciences of the United States of America 78:6517-6521.

Stein MB, Koverola C, Hanna C, Torchia MG, McClarty B (1997) Hippocampal volume in women victimized by childhood sexual abuse. Psychological medicine 27:951-959.

Stenzel-Poore MP, Heinrichs SC, Rivest S, Koob GF, Vale WW (1994) Overproduction of corticotropin-releasing factor in transgenic mice: a genetic model of anxiogenic behavior. The Journal of neuroscience : the official journal of the Society for Neuroscience 14:2579-2584.

Stevens JR, Livermore A, Jr. (1978) Kindling of the mesolimbic dopamine system: animal model of psychosis. Neurology 28:36-46.

Stout SC, Owens MJ, Nemeroff CB (2002) Regulation of corticotropin-releasing factor neuronal systems and hypothalamic-pituitary-adrenal axis activity by stress and chronic antidepressant treatment. The Journal of pharmacology and experimental therapeutics 300:1085-1092.

Strome EM, Wheler GH, Higley JD, Loriaux DL, Suomi SJ, Doudet DJ (2002) Intracerebroventricular corticotropin-releasing factor increases limbic glucose metabolism and has social context-dependent behavioral effects in nonhuman primates. Proceedings of the National Academy of Sciences of the United States of America 99:15749-15754.

Sturm V, Lenartz D, Koulousakis A, Treuer H, Herholz K, Klein JC, Klosterkotter J (2003) The nucleus accumbens: a target for deep brain stimulation in obsessive-compulsive- and anxiety-disorders. Journal of chemical neuroanatomy 26:293-299.

Sun N, Roberts L, Cassell MD (1991) Rat central amygdaloid nucleus projections to the bed nucleus of the stria terminalis. Brain research bulletin 27:651-662.

Sutton RE, Koob GF, Le Moal M, Rivier J, Vale W (1982) Corticotropin releasing factor produces behavioural activation in rats. Nature 297:331-333.

Suzuki T, Shiozaki Y, Masukawa Y, Misawa M, Nagase H (1992) The role of mu- and kappa-opioid receptors in cocaine-induced conditioned place preference. Japanese journal of pharmacology 58:435-442.

Svingos AL, Colago EE, Pickel VM (1999) Cellular sites for dynorphin activation of kappa-opioid receptors in the rat nucleus accumbens shell. The Journal of neuroscience : the official journal of the Society for Neuroscience 19:1804-1813.

Svingos AL, Chavkin C, Colago EE, Pickel VM (2001) Major coexpression of kappa-opioid receptors and the dopamine transporter in nucleus accumbens axonal profiles. Synapse 42:185-192.

Swanson LW (1982) The projections of the ventral tegmental area and adjacent regions: a combined fluorescent retrograde tracer and immunofluorescence study in the rat. Brain research bulletin 9:321-353.

Swanson LW, Simmons DM (1989) Differential steroid hormone and neural influences on peptide mRNA levels in CRH cells of the paraventricular nucleus: a hybridization histochemical study in the rat. The Journal of comparative neurology 285:413-435.

Swanson LW, Sawchenko PE, Rivier J, Vale WW (1983) Organization of ovine corticotropin-releasing factor immunoreactive cells and fibers in the rat brain: an immunohistochemical study. Neuroendocrinology 36:165-186.

Swerdlow NR, Geyer MA, Vale WW, Koob GF (1986) Corticotropin-releasing factor potentiates acoustic startle in rats: blockade by chlordiazepoxide. Psychopharmacology 88:147-152.

Swiergiel AH, Dunn AJ (1999) CRF-deficient mice respond like wild-type mice to hypophagic stimuli. Pharmacology, biochemistry, and behavior 64:59-64.

Swiergiel AH, Leskov IL, Dunn AJ (2008) Effects of chronic and acute stressors and CRF on depression-like behavior in mice. Behavioural brain research 186:32-40.

Takahashi M, Senda T, Tokuyama S, Kaneto H (1990) Further evidence for the implication of a kappa-opioid receptor mechanism in the production of psychological stress-induced analgesia. Japanese journal of pharmacology 53:487-494.

Tallent M, Dichter MA, Bell GI, Reisine T (1994) The cloned kappa opioid receptor couples to an N-type calcium current in undifferentiated PC-12 cells. Neuroscience 63:1033-1040.

Tanaka K, Furuyashiki T, Kitaoka S, Senzai Y, Imoto Y, Segi-Nishida E, Deguchi Y, Breyer RM, Breyer MD, Narumiya S (2012) Prostaglandin E2-mediated attenuation of mesocortical dopaminergic pathway is critical for susceptibility to repeated social defeat stress in mice. The Journal of neuroscience : the official journal of the Society for Neuroscience 32:4319-4329.

Tanapat P, Hastings NB, Rydel TA, Galea LA, Gould E (2001) Exposure to fox odor inhibits cell proliferation in the hippocampus of adult rats via an adrenal hormone-dependent mechanism. The Journal of comparative neurology 437:496-504.

Tang AH, Collins RJ (1985) Behavioral effects of a novel kappa opioid analgesic, U-50488, in rats and rhesus monkeys. Psychopharmacology 85:309-314.

Tao R, Auerbach SB (2002) Opioid receptor subtypes differentially modulate serotonin efflux in the rat central nervous system. The Journal of pharmacology and experimental therapeutics 303:549-556.

Tejeda HA, Shippenberg TS, Henriksson R (2012) The dynorphin/kappa-opioid receptor system and its role in psychiatric disorders. Cellular and molecular life sciences : CMLS 69:857-896.

Tejeda HA, Chefer VI, Zapata A, Shippenberg TS (2010a) The effects of kappa-opioid receptor ligands on prepulse inhibition and CRF-induced prepulse inhibition deficits in the rat. Psychopharmacology 210:231-240.

Tejeda HA, Schultz K, Chefer VI, Shippenberg TS (2010b) Modulation of mesocortical dopamine transmission by mu- and kappa-opioid receptors. Society for Neuroscience Abstracts:368.324.

Thierry AM, Tassin JP, Blanc G, Glowinski J (1976) Selective activation of mesocortical DA system by stress. Nature 263:242-244.

Thompson AC, Zapata A, Justice JB, Jr., Vaughan RA, Sharpe LG, Shippenberg TS (2000) Kappa-opioid receptor activation modifies dopamine uptake in the nucleus accumbens and opposes the effects of cocaine. The Journal of neuroscience : the official journal of the Society for Neuroscience 20:9333-9340.

Tidey JW, Miczek KA (1996) Social defeat stress selectively alters mesocorticolimbic dopamine release: an in vivo microdialysis study. Brain research 721:140-149.

Timpl P, Spanagel R, Sillaber I, Kresse A, Reul JM, Stalla GK, Blanquet V, Steckler T, Holsboer F, Wurst W (1998) Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nature genetics 19:162-166.

Tischmeyer W, Grimm R (1999) Activation of immediate early genes and memory formation. Cellular and molecular life sciences : CMLS 55:564-574.

Todtenkopf MS, Marcus JF, Portoghese PS, Carlezon WA, Jr. (2004) Effects of kappa-opioid receptor ligands on intracranial self-stimulation in rats. Psychopharmacology 172:463-470.

Tomasiewicz HC, Todtenkopf MS, Chartoff EH, Cohen BM, Carlezon WA, Jr. (2008) The kappa-opioid agonist U69,593 blocks cocaine-induced enhancement of brain stimulation reward. Biological psychiatry 64:982-988.

Toth Z, Hollrigel GS, Gorcs T, Soltesz I (1997) Instantaneous perturbation of dentate interneuronal networks by a pressure wave-transient delivered to the neocortex. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:8106-8117.

Tsuchiya K, Inoue T, Koyama T (1996) Effect of repeated methamphetamine pretreatment on freezing behavior induced by conditioned fear stress. Pharmacology, biochemistry, and behavior 54:687-691.

Turgeon SM, Pollack AE, Fink JS (1997) Enhanced CREB phosphorylation and changes in c-Fos and FRA expression in striatum accompany amphetamine sensitization. Brain research 749:120-126.

Ungless MA, Singh V, Crowder TL, Yaka R, Ron D, Bonci A (2003) Corticotropin-releasing factor requires CRF binding protein to potentiate NMDA receptors via CRF receptor 2 in dopamine neurons. Neuron 39:401-407.

Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, Miller KJ, Spedding M, Mailman RB (2007) Functional selectivity and classical concepts of quantitative pharmacology. The Journal of pharmacology and experimental therapeutics 320:1-13.

Valdez GR, Zorrilla EP, Rivier J, Vale WW, Koob GF (2003) Locomotor suppressive and anxiolytic-like effects of urocortin 3, a highly selective type 2 corticotropin-releasing factor agonist. Brain research 980:206-212.

Vale W, Spiess J, Rivier C, Rivier J (1981) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 213:1394-1397.

Valenti O, Lodge DJ, Grace AA (2011) Aversive stimuli alter ventral tegmental area dopamine neuron activity via a common action in the ventral hippocampus. The Journal of neuroscience : the official journal of the Society for Neuroscience 31:4280-4289.

Valjent E, Bertran-Gonzalez J, Aubier B, Greengard P, Herve D, Girault JA (2010) Mechanisms of locomotor sensitization to drugs of abuse in a two-injection protocol. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 35:401-415.

Van't Veer A, Carroll FI, Carlezon Jr. WA (2011) Antagonism of kappa-opioid receptors reduces corticotropin-releasing factor induced effects. In: Society for Neuroscience, p Program Number 791.710. Washington, D.C.

Van't Veer A, Yano JM, Carroll FI, Cohen BM, Carlezon WA, Jr. (2012) Corticotropin-Releasing Factor (CRF)-Induced Disruption of Attention in Rats Is Blocked by the kappa-Opioid Receptor Antagonist JDTic. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 37:2809-2816.

van Gaalen MM, Stenzel-Poore MP, Holsboer F, Steckler T (2002) Effects of transgenic overproduction of CRH on anxiety-like behaviour. The European journal of neuroscience 15:2007-2015.

Van Pett K, Viau V, Bittencourt JC, Chan RK, Li HY, Arias C, Prins GS, Perrin M, Vale W, Sawchenko PE (2000) Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. The Journal of comparative neurology 428:191-212.

Vanderwolf CH (1992) Hippocampal activity, olfaction, and sniffing: an olfactory input to the dentate gyrus. Brain research 593:197-208.

Vaught JL, Takemori AE (1979) Differential effects of leucine and methionine enkephalin on morphine-induced analgesia, acute tolerance and dependence. The Journal of pharmacology and experimental therapeutics 208:86-90.

Viengchareun S, Le Menuet D, Martinerie L, Munier M, Pascual-Le Tallec L, Lombes M (2007) The mineralocorticoid receptor: insights into its molecular and (patho)physiological biology. Nuclear receptor signaling 5:e012.

Villarreal G, Hamilton DA, Petropoulos H, Driscoll I, Rowland LM, Griego JA, Kodituwakku PW, Hart BL, Escalona R, Brooks WM (2002) Reduced hippocampal volume and total white matter volume in posttraumatic stress disorder. Biological psychiatry 52:119-125.

Vincent S, Hokfelt T, Christensson I, Terenius L (1982) Immunohistochemical evidence for a dynorphin immunoreactive striato-nigral pathway. European journal of pharmacology 85:251-252.

Volkow ND, Li TK (2004) Drug addiction: the neurobiology of behaviour gone awry. Nature reviews Neuroscience 5:963-970.

Vrana SR, Spence EL, Lang PJ (1988) The startle probe response: a new measure of emotion? Journal of abnormal psychology 97:487-491.

Walker BM, Valdez GR, McLaughlin JP, Bakalkin G (2012) Targeting dynorphin/kappa opioid receptor systems to treat alcohol abuse and dependence. Alcohol 46:359-370.

Walker DL, Davis M (1997) Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle increases produced by conditioned versus unconditioned fear. The Journal of neuroscience : the official journal of the Society for Neuroscience 17:9375-9383.

Walsh TJ, Schulz DW, Tilson HA, Schmechel DE (1986) Colchicine-induced granule cell loss in rat hippocampus: selective behavioral and histological alterations. Brain research 398:23-36.

Wanat MJ, Hopf FW, Stuber GD, Phillips PE, Bonci A (2008) Corticotropin-releasing factor increases mouse ventral tegmental area dopamine neuron firing through a protein kinase C-dependent enhancement of Ih. The Journal of physiology 586:2157-2170.

Wang B, Shaham Y, Zitzman D, Azari S, Wise RA, You ZB (2005) Cocaine experience establishes control of midbrain glutamate and dopamine by corticotropin-releasing factor: a role in stress-induced relapse to drug seeking. The Journal of neuroscience : the official journal of the Society for Neuroscience 25:5389-5396.

Wang YJ, Rasakham K, Huang P, Chudnovskaya D, Cowan A, Liu-Chen LY (2011) Sex difference in kappa-opioid receptor (KOPR)-mediated behaviors, brain region KOPR level and KOPR-mediated guanosine 5'-O-(3-[35S]thiotriphosphate) binding in the guinea pig. The Journal of pharmacology and experimental therapeutics 339:438-450.

Warnock G, Moechars D, Langlois X, Steckler T (2009) In vivo evidence for ligand-specific receptor activation in the central CRF system, as measured by local cerebral glucose utilization. Peptides 30:947-954.

Warren BL, Vialou VF, Iniguez SD, Alcantara LF, Wright KN, Feng J, Kennedy PJ, Laplant Q, Shen L, Nestler EJ, Bolanos-Guzman CA (2013) Neurobiological sequelae of witnessing stressful events in adult mice. Biological psychiatry 73:7-14.

Weber E, Barchas JD (1983) Immunohistochemical distribution of dynorphin B in rat brain: relation to dynorphin A and alpha-neo-endorphin systems. Proceedings of the National Academy of Sciences of the United States of America 80:1125-1129.

Weber E, Roth KA, Barchas JD (1982) Immunohistochemical distribution of alpha-neo-endorphin/dynorphin neuronal systems in rat brain: evidence for colocalization. Proceedings of the National Academy of Sciences of the United States of America 79:3062-3066.

Wee S, Koob GF (2010) The role of the dynorphin-kappa opioid system in the reinforcing effects of drugs of abuse. Psychopharmacology 210:121-135.

Werling LL, Frattali A, Portoghese PS, Takemori AE, Cox BM (1988) Kappa receptor regulation of dopamine release from striatum and cortex of rats and guinea pigs. The Journal of pharmacology and experimental therapeutics 246:282-286.

West AR, Floresco SB, Charara A, Rosenkranz JA, Grace AA (2003) Electrophysiological interactions between striatal glutamatergic and dopaminergic systems. Annals of the New York Academy of Sciences 1003:53-74.

Whitnall MH, Smyth D, Gainer H (1987) Vasopressin coexists in half of the corticotropin-releasing factor axons present in the external zone of the median eminence in normal rats. Neuroendocrinology 45:420-424.

Widerlov E, Bissette G, Nemeroff CB (1988) Monoamine metabolites, corticotropin releasing factor and somatostatin as CSF markers in depressed patients. Journal of affective disorders 14:99-107.

Wignall EL, Dickson JM, Vaughan P, Farrow TF, Wilkinson ID, Hunter MD, Woodruff PW (2004) Smaller hippocampal volume in patients with recent-onset posttraumatic stress disorder. Biological psychiatry 56:832-836.

Wiley MD, Poveromo LB, Antapasis J, Herrera CM, Bolanos Guzman CA (2009) Kappa-opioid system regulates the long-lasting behavioral adaptations induced by early-life exposure to methylphenidate. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 34:1339-1350.

Wilkinson LS, Humby T, Killcross AS, Torres EM, Everitt BJ, Robbins TW (1998) Dissociations in dopamine release in medial prefrontal cortex and ventral striatum during the acquisition and extinction of classical aversive conditioning in the rat. The European journal of neuroscience 10:1019-1026.

Wise RA, Bozarth MA (1987) A psychomotor stimulant theory of addiction. Psychological review 94:469-492.

Wise RA, Rompre PP (1989) Brain dopamine and reward. Annual review of psychology 40:191-225.

Wittmann W, Schunk E, Rosskothen I, Gaburro S, Singewald N, Herzog H, Schwarzer C (2009) Prodynorphin-derived peptides are critical modulators of anxiety and regulate neurochemistry and corticosterone. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 34:775-785.

Wood SK, McFadden KV, Grigoriadis D, Bhatnagar S, Valentino RJ (2012) Depressive and cardiovascular disease comorbidity in a rat model of social stress: a putative role for corticotropin-releasing factor. Psychopharmacology.

Woods JH, Katz JL, Winger G (1992) Benzodiazepines: use, abuse, and consequences. Pharmacological reviews 44:151-347.

Wynn PC, Hauger RL, Holmes MC, Millan MA, Catt KJ, Aguilera G (1984) Brain and pituitary receptors for corticotropin releasing factor: localization and differential regulation after adrenalectomy. Peptides 5:1077-1084.

Xu Y, Day TA, Buller KM (1999) The central amygdala modulates hypothalamic-pituitary-adrenal axis responses to systemic interleukin-1beta administration. Neuroscience 94:175-183.

Yadid G, Overstreet DH, Zangen A (2001) Limbic dopaminergic adaptation to a stressful stimulus in a rat model of depression. Brain research 896:43-47.

Yeomans JS, Frankland PW (1995) The acoustic startle reflex: neurons and connections. Brain research Brain research reviews 21:301-314.

Yeomans JS, Li L, Scott BW, Frankland PW (2002) Tactile, acoustic and vestibular systems sum to elicit the startle reflex. Neuroscience and biobehavioral reviews 26:1-11.

Young AM, Joseph MH, Gray JA (1993) Latent inhibition of conditioned dopamine release in rat nucleus accumbens. Neuroscience 54:5-9.

Zacharko RM, Anisman H (1991) Stressor-induced anhedonia in the mesocorticolimbic system. Neuroscience and biobehavioral reviews 15:391-405.

Zhang S, Tong Y, Tian M, Dehaven RN, Cortesburgos L, Mansson E, Simonin F, Kieffer B, Yu L (1998) Dynorphin A as a potential endogenous ligand for four members of the opioid receptor gene family. The Journal of pharmacology and experimental therapeutics 286:136-141.

Zhang Y, Butelman ER, Schlussman SD, Ho A, Kreek MJ (2005) Effects of the plant-derived hallucinogen salvinorin A on basal dopamine levels in the caudate putamen and in a conditioned place aversion assay in mice: agonist actions at kappa opioid receptors. Psychopharmacology 179:551-558.

Zhu J, Chen C, Xue JC, Kunapuli S, DeRiel JK, Liu-Chen LY (1995) Cloning of a human kappa opioid receptor from the brain. Life sciences 56:PL201-207.

Ziegler DR, Herman JP (2002) Neurocircuitry of stress integration: anatomical pathways regulating the hypothalamo-pituitary-adrenocortical axis of the rat. Integrative and comparative biology 42:541-551.

Zobel AW, Nickel T, Kunzel HE, Ackl N, Sonntag A, Ising M, Holsboer F (2000) Effects of the high-affinity corticotropin-releasing hormone receptor 1 antagonist R121919 in major depression: the first 20 patients treated. Journal of psychiatric research 34:171-181.

Zorrilla EP, Koob GF (2010) Progress in corticotropin-releasing factor-1 antagonist development. Drug discovery today 15:371-383.